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A study of selected algal-bacterial interactions.

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A STUDY OF SELECTED ALGAL-BACTERIAL INTERACTIONS

A Dissertation Presented

By

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ABSTRACT

A Study of Selected Algal-Bacterial Interactions

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The interactions between several green algae and an Arthrobacter sp. isolated from a lake were observed. When inoculated together under light in a mineral salt solution (no exogenous organic carbon), Chlorella suppressed the bacteria; the degree of inhibition was proportional to initial algal numbers. Inhibition was reversed by the addition of mineral salts, especially when the pH was below 8.6. In contrast to Chlorella, actively growing Ankistrodesmus cells strongly stimulated the Arthrobacter sp. in mineral salt solution, while actively growing Chlamydomonas slightly enhanced bacterial growth. Actively growing Chlorococcum, however, failed to affect Arthrobacter growth significantly. Chlorococcum filtrate stimulated bacterial growth; the older the filtrate, the greater the enhancement.

Filtrates of Chlorella, Ankistrodesmus, Chlorococcum, and Chlamydomonas all stimulated the growth of three bacter-

ial species growing together, but no filtrate was either more or less stimulatory than any other. The Nocardia sp. was enhanced somewhat less than either the Flavobacterium sp. or the Arthrobacter sp. in all four filtrates. It therefore appears that active cells of the green algae tested affect lake bacteria in a variety of ways, but their filtrates are generally stimulatory, the degree of stimulation varying with respective bacteria. In solutions low in organic matter, Chlorella inhibited Arthrobacter by more efficiently competing for inorganic nutrients at high pH values.

In contrast, the Arthrobacter sp. did not affect Chlorella growth in a liquid mineral salts medium, but actively growing cells did suppress algal growth on an organic solid medium. Bacterial cells which had been disrupted, boiled, or treated with chloroform did not suppress Chlorella growth. Live Flavobacterium or Nocardia cells did not inhibit Chlorella on a solid medium.

A number of tests were conducted to determine the cause of suppression of Chlorella by the Arthrobacter sp. Competitive inhibition for nutrients was not found to be responsible, and no evidence of enzyme activity was apparent. Several other heterotrophic bacteria isolated from the lacustrine environment displayed the same inhibition pattern when streaked on a Chlorella-inoculated agar plate. For these organisms and the Arthrobacter sp., a positive correla-

tion was noted between algal inhibition on these plates and the ability of a bacterium to nitrify. Ammonia, nitrite, nitrate, and nitropropane at a concentration of 10 µg/ml sample-N did not suppress algal growth, but hydroxylamine and, to a lesser extent, oxime did. The zone of inhibition on agar plates contained over 5 µg/ml hydroxylamine-N four days after streaking, while areas of algal growth on these same plates were found to contain a maximum of only 0.2 µg/ml hydroxylamine-N. Almost no hydroxylamine was found in the areas of algal growth until the fourth day after the appearance of algal colonies. In mineral salts medium (pH 6.6), 0.16 µg/ml hydroxylamine-N was established as an upper limit for Chlorella growth. Chemical inhibitors of autotrophic nitrification failed to suppress heterotrophic nitrification.

In other tests, very high levels of glucose (3% or more) significantly decreased the zone of inhibition on plates, possibly due to a corresponding increase in nitrogen assimilation by the nitrifying bacterium. The decrease was not due to Chlorella stimulation by the extra glucose. On Chlorella-inoculated agar plates containing 12 µg/ml hydroxylamine-N and streaked once with a hydroxylamine-degrading bacterium, Chlorella colonies appeared only in the close proximity of the bacterial streak. It thus appears that hydroxylamine is the toxic principle of Chlorella inhibition.

In summary, the results of this investigation indi-

cate that the interactions of *Chlorella* and *Arthrobacter* depend very strongly on the level of available inorganic and organic nutrients. The manipulation of the nutrient balance to favor heterotrophic nitrification may have significant use in the biological control of algal blooms.

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INTRODUCTION

The effects of urbanization on aquatic ecosystems are only known in general terms. Nutrient enrichment from urban discharge accelerates eutrophication and often results in excessive growth of algae. An algal bloom may kill fish and other wildlife, create noxious odors, impair aesthetics and taste, clog filters, and obstruct recreation. Thus, the effects of urban discharge may pose a threat to water quality and to the health of a community.

In addition to the obvious effects of urban discharge, the resulting nutrient enrichment of lakes has great impact on the population dynamics of the microbial ecosystem. This is especially true of nitrogen compounds. Of the many transformations undergone by nitrogen in lakes, one of the more important is that of nitrification, the biological oxidation of reduced nitrogen compounds. When performed by a heterotrophic organism, the term heterotrophic nitrification is used. The byproducts of heterotrophic nitrification may include substances which are known growth factors (e.g., some of the hydroxamic acids), others which are toxic and even mutagenic (e.g., hydroxylamine and nitroso compounds), and still other compounds which can serve as a source of nitrogen for assimilation (e.g., nitrate). Their effect on the aquat-

ic ecosystem and specifically on the pattern of algal blooms is virtually unknown.

Attempts to control algal blooms by mechanical and chemical means have often failed, mainly due to the elementary state of our knowledge of the dynamics of the microbial ecosystem. A more useful approach for minimizing excessive algal growth, given that nutrient loading continues, is the control of these blooms by biological means. Yet such a regimen would require a particularly profound understanding of the population dynamics of an aquatic ecosystem. While a diverse and widely-based literature exists on the biological control of eutrophication (for a review, see Schuytema, 1977), information on the relationship between bacteria and algae is very meager. This thesis will attempt to deal with this question.

In this investigation, two broad areas were examined: the effects of various green algae on the growth of bacteria, and the response of a particular alga to a bacterium isolated from a lake. It will be seen that the inorganic and organic nutrient levels are critical to the algal-bacterial interaction, and that a product of heterotrophic nitrification produced by the bacterium has great impact on this association.

LITERATURE REVIEW

Effects of Algae on Bacteria

Almost all of the investigations performed to date focus on the effect of algae on bacteria, with only a few dwelling on the reverse relationship. A general statement on the effect of algae on bacteria cannot be made, since there is little agreement on the subject in the literature. Chrost (1975) found that elimination and selection of bacteria occurred during algal blooms in an eutrophic lake. The bloom-forming algae inhibited gram-positive but not gram-negative bacteria. Overall a strong decrease in the number of bacteria in the lake occurred, and indeed organic matter degradation in the euphotic zone was inhibited. McCoy and Sarles (1969) also noted the great predominance of gram-negative bacteria in lakes, and suggested that selection was occurring. In tests of the antibacterial activity of the North Sea, Moebus (1972a) observed that the breakdown of phytoplankton blooms produced marked inhibition of Escherichia coli, Serratia marinoruba, and Staphylococcus aureus. In contrast to these findings, Jones (1972), Blasco (1965), Silvey and Wyatt (1969), and Ganapati (1975) found a positive correlation in freshwater lakes between bacterial counts and numbers of several types of green and blue-green

algae. In a southwestern U.S. reservoir, Silvey and Wyatt (1969) noted that occurrence of blue-green algal blooms led to an increase of actinomycetes and gram-positive bacteria. In variance with the previous findings, Sieburth (1968) noted the bacterial flora in Narragansett Bay was apparently not influenced by marked seasonal outbursts in phytoplankton. He suggested that the lack of correlation may be due to the influence of ciliates and other organisms. In addition, no correlation was noted between phytoplankton and bacterial numbers by Saunders (1969) in two Michigan lakes, and by Menon et al. (1972) in Lake Erie. The lack of consistent observations in the previously-mentioned studies is undoubtedly due to strain variation and numerous environmental factors.

It is well established that actively growing algae can release into the surrounding water part of their photosynthate. Some of the products are known to stimulate heterotrophs, while several have been shown to be inhibitory. Environmental factors influence the relationship. Algal stimulation of bacteria has been studied by a number of investigators. Great variation has been found in the percentage of organic matter released to that synthesized. In one of the first investigations, Krogh and Lange (1930) found evidence that Scenedesmus loses to the surrounding water at most 10% of the organic material synthesized. They attributed these losses to dead and decomposed organisms.

Many of the recent studies in determining percentage

release have used carbon-14 tracers. Belly et al. (1973) found that in an acid spring the eucaryotic alga Cyanidium caldarium secreted 2-6% of its organic carbon, while Hellebust (1965) found a similar secretion rate in a number of species of unicellular marine algae. In the latter study some algae were able to secrete 10-25% of their carbon. Allen (1969) reported that 65% of the fixed carbon of the emergent plant Scirpus acustus was released into lakewater over a four-hour period, and that epiphytes removed much of the labeled material. In an often-quoted paper Fogg et al. (1965) found that living phytoplankton cells released between 7 and 50% of the total carbon fixed, and occasionally 95% was released. In a very recent article, Sharp (1977) strongly argued that improper use of the carbon-14 technique normally employed in such studies invalidated most of the findings, a charge that Fogg (1977) disputes, at least with regard to his own investigation (Fogg, 1965). In an in situ study, Antia et al. (1963) induced a bloom in a large floating plastic sphere and reported that phytoplankton cells excreted 35-40% of their organic matter during growth. Their data suggested that this matter was released chiefly from live cells.

The extracellular release of a variety of substances by algae is well-established. Several recent reviews of this topic are available (Fogg, 1962, 1966, 1971; Hellebust, 1974). Types of products found include glycollic acid and

other organic acids, various mono and disaccharides, polysaccharides, sugar alcohols, amino acids, peptides, proteins, lipids, vitamins, enzymes, phenolic compounds, and various types of toxins. Glycollic acid has been the most widely investigated extracellular substance involving algae, and probably represents the major product excreted by these organisms. Hellebust (1965), for example, found this organic acid in the filtrates of all but one of the twenty-three species of marine algae investigated. Glycollic acid is the primary substrate in the process of photorespiration, defined as light-dependent oxygen uptake and carbon dioxide release (Tolbert, 1974). The reason for glycollate release is currently unknown, but certainly there has been much speculation in the literature (Odum et al., 1969; Lange, 1971; Krauss, 1962; Sieburth, 1968).

The concentration of several substances known to be excreted by algae has been measured in both marine and fresh water. Glycollic acid concentrations ranged from 0 to 60 µg/l in both fresh waters (Fogg et al., 1969) and marine waters (Al-Hasan et al., 1975). Spear and Lee (1968) failed to detect glycollate in Wisconsin lakes. The concentrations of glucose and sucrose were 1 mg/m^3 and 2 mg/m^3, respectively, in an oligotrophic lake and 2 mg/m^3 and 5 mg/m^3, respectively, in a eutrophic lake (Vallentyne and Whittaker, 1956). In this study, a greater concentration with depth in the eutrophic lake was noted. Glucose levels in the Atlantic Ocean

vary from 10^{-6}M to values below 10^{-8}M (Vaccaro et al., 1968). The concentration of this sugar exhibited a positive correlation with phytoplankton density. Vitamin B_{12} has also been measured in seawater and was found to vary with time of year and depth (Menzel and Spaeth, 1962). Concentrations ranged from about 0.01 to almost 0.5 mμg/l.

Several environmental factors have been found to influence both percentage organic release and types of products excreted. These include age of culture (Nalewajko and Lean, 1972), density of culture (Watt, 1966), intensity of sunlight (Watt, 1966; Hellebust, 1965; Fogg et al., 1965), and carbon dioxide concentration (Watt and Fogg, 1966). Using Sephadex fractionation, Nalewajko and Lean (1972) found a shift from small molecular weight particles to larger molecular weight compounds as monocultures of common lake algae aged. They also found that percentage release values increased with increasing growth rates in short-term experiments in three of four strains of algae studied. In contrast, Hellebust (1974), in his review of extracellular products of algae, cited several articles that generally indicate a greater release of organic carbon during lag and stationary growth phases.

Besides culture age, culture density was found to alter percentage release. The lower the density (or greater the dilution), the greater was the percent release per cell (Watt, 1966). Thus phytoplankton in oligotrophic lakes excrete a higher percentage of the carbon fixed than in more

productive waters (Wetzel, 1975). This result may be due to the greater osmotic gradient between algal organic substance and the more dilute oligotrophic water.

High light intensities result in an increase of percent release (Watt, 1966; Fogg et al., 1965), possibly due to membrane damage. There is a decrease, however, in the absolute amount of glycollate secreted per cell (Fogg, 1971). Low light intensities may also increase percent release. Watt (1966) found that percent release increases with depth. Watt and Fogg (1966) investigated all possible combinations of light intensity and carbon dioxide concentrations and found the greatest release of glycollate occurred when Chlorella pyrenoidosa grown under low light and high carbon dioxide conditions was suddenly transferred to high light and low carbon dioxide. After 50 to 100 minutes, the rate of glycollate release decreased, suggesting to the investigators an exhaustion of the glycollate precursor. In a later paper, Fogg (1977) suggested that the combination of a lack of essential nutrients and a high photosynthate may cause excretion.

Thus it has been established that algae excrete a variety of extracellular products into the surrounding water, and that the percentage release and type of products vary according to environmental factors. Several lines of evidence indicate that these products can be used by the indigenous bacterial population for growth.

Investigators have added carbon-14 labeled substrates known to be excreted by algae to natural populations of planktonic bacteria and have measured a significant rate of uptake (Williams and Gray, 1970; Allen, 1969; Nalewajko and Lean, 1972; Wright, 1975). Nalewajko and Lean (1972), for example, demonstrated that bacteria use low molecular weight extracellular products such as glycollate and, in turn, form larger molecular weight compounds. In a similar study, Berland et al. (1970) tested 77 organic compounds for susceptibility to attack by 25 species of bacteria isolated from marine algal cultures. These investigators found that bacteria used amino acids and organic acids more frequently than sugars and sugar derivatives, especially when vitamins were lacking. Using Adler's method for demonstrating chemotaxis, Bell and Mitchell (1972) also found that specific compounds known to occur as algal extracellular products attracted marine bacterial isolates. In this experiment, however, the threshold concentrations for attraction were unexpectedly high when compared to the generally low organic concentrations found in natural sea water.

Another line of evidence that bacteria used algal extracellular products involves measurements on the rate of accumulation of various organic compounds released by algae in water (Hobbie, 1969; Spear and Lee, 1968; Nalewajko et al., 1975; Gocke, 1970). Spear and Lee (1968) found no glycollate in Madison, Wisconsin lakes over a two-year per-

iod. Yet six strains of Chlorella pyrenoidosa did release glycollate in culture medium. In another investigation, extracellular release from the alga Chlorella pyrenoidosa Chick was measured in both axenic cultures and cultures in which bacteria had been added (Nalewajko et al., 1976). In the axenic algal culture, extracellular release was linear with time; in the mixed culture, plateau-type curves were obtained. Gocke (1970) reported that the accumulation in the culture medium of dissolved organic material, and especially dissolved amino compounds, from the alga Scenedesmus quadricauda was much smaller in contaminated cultures than in bacteria-free cultures.

The close physical association of algae and bacteria is a suggestion of another line of evidence. Electron micrographs of freshwater samples containing two blue-green algal species capable of nitrogen fixation revealed that bacteria were attached specifically at the polar regions of heterocysts, the sites of nitrogen fixation (Paerl, 1976).

Some of the metabolites excreted have been found to act as chelating substances (Kroes, 1972). This could benefit bacterial growth. Oxygen resulting from algal growth undoubtedly also contributes to bacterial growth. Ganapati (1975) describes a symbiotic relationship between algae and bacteria in sewage oxidation ponds, where blue-green and green algae product oxygen, which in turn is used by bacteria to degrade organic material.

Algae can also inhibit bacteria, as alluded to previously. There is evidence that this antagonism may either involve competitive inhibition or toxins released from the algal cell. Regarding the former, Fitzgerald (1969) found that aquatic weeds and some filamentous green algae remained relatively free of epiphytes, possibly due to the plant's ability to act as a nitrogen sink. Yet Wright and Hobbie (1966) and Rhee (1972) provide evidence that bacteria are substantially more efficient than algae in the uptake of at least some nutrients.

It is well known that some algae can produce toxins effective against bacteria. Shilo (1967) identified certain of these as endotoxins (e.g., Gymnodinium breve and all toxigenic blue-green algae), while others are exotoxins (e.g., Prymnesium parvum). The bactericidal activity of seawater is well known (Shilo, 1967; Saz et al., 1963). Marine algal samples from Puerto Rico (Burkholder et al., 1960), Great Britain (Hornsey and Hide, 1974), the Weddell Sea (Sieburth, 1959), and Vineyard Sound, Massachusetts (Saz et al., 1963) were shown to display antibiotic activity. Of the 150 algae from Puerto Rico, 66 inhibited Staphylococcus aureus and other laboratory microbes, and a few algae strongly inhibited marine bacteria. The dinoflagellate Gonyaulax tamarensis, one of the causative organisms of the red tide, revealed both promotion and inhibition activity for crude populations of marine bacteria (Burkholder et al., 1960). The marine phyto-

flagellate Prymnesium parvum, which inhabits brackish water, also produces a toxin (Shilo, 1967).

In other investigations, cultures of Chlorella vulgaris and Chlorella pyrenoidosa were found to accumulate a substance which was toxic to both gram-positive and gram-negative bacteria, including Pseudomonas aeruginosa (Pratt et al., 1944). Confirming this finding, Vela and Guerra (1966) demonstrated that Chlorella pyrenoidosa inhibited the growth of many species of bacteria, including human pathogens and most soil and air bacteria. Moreover, those species inhibited by the live algae could not grow in algae filtrates. It is known that Chlorella vulgaris liberates a substance which retards its own growth (Pratt and Fong, 1940), probably a peroxide produced by the photo-oxidation of unsaturated fatty acids (Scutt, 1964).

Cell extracts of algae have also been shown in some cases to be toxic to bacteria. Extracts of the marine gulfweed Sargassum natans inhibited a Pseudomonas strain isolated from fouled weeds (Sieburth and Conover, 1965). Duff et al. (1966) demonstrated the antibacterial activity of cell extracts of fourteen species of marine phytoplankters. Representatives of the Bacillariophyceae, Chrysophyceae, and Cryptophyceae showed a significantly greater range and activity than the two Chlorophycean species, and greatest activity was evident against marine isolates and gram-positive bacteria. They also found evidence that some bacteria were

stimulated by products secreted by the toxic algae. In some cases, healthy algae are relatively free of bacteria. This is true of Sargassum natans (Sieburth and Conover, 1965) and the diatom Skeletonema (Droop and Elson, 1966).

Little information has been published on the identity of bacteria-inhibiting compounds produced by algae, and it is almost exclusively limited to studies with marine algae. Acrylic acid was isolated from the colonial alga Phaeocystis which is active against Staphylococcus aureus and Mycobacterium smegmatis (Sieburth, 1960). This alga, when digested by penguins, also inhibits the normal gastrointestinal microflora of these creatures. In another study, tannic acid from Sargassum natans inhibited a Pseudomonas strain (Sieburth and Conover, 1965). Katayama (1962) found that several volatile constituents from algae (fatty acids, carbonyl, and terpene) inhibited Staphylococcus aureus and Escherichia coli.

Saz et al. (1963) demonstrated that the active factor inhibiting Staphylococcus aureus (but not coliforms) was a large, non-dialyzable autoclave-labile molecule. Berland et al. (1972a) isolated and purified two substances from the marine alga Stichochrysis immobilis which demonstrated antibacterial activity. One was a peptide (molecular weight about 3400); the other substance was not identified.

A number of environmental factors can contribute to the antibacterial activity of an established algal popula-

tion. Maksimova and Fedenko (1965) found that Chlorella vulgaris was toxic for Bacillus cereus and Pseudomonas ovalis only under high redox potentials. Moebus (1972b) demonstrated that very low amounts of organic carbon added to sterile seawater increased toxicity of that water against Escherichia coli, Staphylococcus aureus, and Serratia marinorubra. In a study involving Prymnesium parvum, Shilo (1967) found that lack of light eliminated toxin synthesis, changes in pH inactivated the toxin, and phosphate limitation enhanced toxin formation.

The aforementioned investigations demonstrate unequivocally that algae influence the growth of a variety of bacteria. Indeed a particular algal population may stimulate some strains of bacteria more than others and the closer the bacterium is to the algal cells, the greater the effect. Bell and Mitchell (1972) investigated this phenomenon in the marine environment and coined the term "physosphere" to describe a zone extending outward from an algal cell or colony in which bacterial growth is stimulated by extracellular products of the alga. These investigators found evidence that specific bacteria were selected from a mixed culture by algal extracellular products. In a later publication (Bell et al., 1974), they demonstrated that two marine bacteria (Pseudomonas HNY and Spirillum 7697) differed considerably in their uptake rates of extracellular products produced by the alga Skeletonema costatum.

By examination of the concentrations of various organic fractions, Maksimova and Pimenova (1969b) concluded that different bacteria used different substrates when grown with several Chlorella species. Flavobacterium diffusum consumed free sugars and volatile acids of algal filtrates; polysaccharides were not used. In contrast Pseudomonas pyocyanea failed to consume free sugars in the medium but did metabolize the polysaccharides. Sieburth (1968) reported that at diatom peaks in Narragansett Bay, Vibrio was inhibited, while Flavobacterium dominated.

In general, gram-negative organisms predominate over gram-positive organisms in both freshwater and seawater, indicating that a selection process is occurring (Chrost, 1975; Saz et al., 1963). Berland et al. (1969) isolated many bacterial strains from marine algal cultures and identified them. They found that in general Pseudomonas, Flavobacterium, and Achromobacter appeared to be the most important as far as the number of species was concerned. Other genera found included Vibrio, Agarbacterium, Xanthomonas, Micrococcus, and Staphylococcus. On a broader level, the well-established principle that non-indigenous microflora cannot successfully compete with well-adapted native forms undoubtedly serves to restrict growth in the aquatic milieu to a relatively small number of bacterial species.

Effects of Bacteria on Algae

Far fewer articles have been published on the effect of bacteria on algae. Bacteria are known to enhance algal growth in at least a few cases, and inhibit them in others. The few articles dealing with algal stimulation usually involve the production by bacteria of vitamins and carbon dioxide. A number of investigators have shown that the growth rates of some algae are stimulated when thiamine, biotin or especially vitamin B_{12} is added to culture medium (Wetzel, 1975; Carlucci and Bowes, 1970). Gordon et al. (1969) demonstrated that Chlorella was dependent upon bacteria for thiamine, as well as carbon dioxide and possibly nitrogen.

Other investigators have shown at least in vitro, that carbon dioxide stimulates algal growth. Garrett (1975) reported that algal productivity in mixed algal-bacterial communities was greater than that in axenic systems, and this was attributed to carbon dioxide produced by bacteria. The addition of this gas to an axenic algal system significantly reduced the rate of glycollate excretion. In several articles, Lange (1967, 1970, 1971) reported algal growth enhancement in mixed algal-bacterial cultures as opposed to axenic algal cultures when organic carbon was added to both systems. He concluded that the organic addition not only resulted in bacterial metabolism yielding carbon dioxide, but also delayed bacterial assimilation of organic chelating

agents needed to keep iron and trace elements available to the algae.

Whether the carbon dioxide enhancement of algal growth occurs in nature is uncertain. In a study of a freshwater lake in England, indirect estimates of carbon dioxide evolvement by bacteria were much lower than similar estimates of the net uptake of the algae. Thus the bacteria could not significantly stimulate the algae (Jones, 1972). Possibly algal stimulation may occur in lakes with high pH.

Besides the production of vitamins and carbon dioxide, bacteria have been found to stimulate algae in other ways. It is well known that mineralization is a vital process in recycling inorganic substances required for algal growth (Sieburth, 1968). Perhaps bacterially-derived organic substances can also stimulate algal growth. Numerous algae have been found to supplement photoautotrophy by the uptake and use of organic substances, and in some cases, these substances are growth-essential (Wetzel, 1975). In addition, a large number of algae can grow heterotrophically in the dark (see reviews of algal heterotrophy by Neilson and Lewin, 1974; Droop, 1974). In fact the process may be widespread. Fourteen of twenty filamentous blue-green algae grew in the dark when supplied with simple sugars (Khoja and Whitton, 1975). Algae living heterotrophically can use only a limited range of substrates and these include gelatins (Beijerinck, 1898), glucose (Algeus, 1948), fructose (Khoja

and Whitton, 1975), sucrose (Khoja and Whitton, 1975), maltose (Bennett and Hobbie, 1972), galactose (Samejima and Myers, 1958), beta-glucosides (Neish, 1951), and acetate (Samejima and Myers, 1958).

In other studies on bacterial stimulation of algae, Ukeles and Bishop (1975) concluded that algal stimulation observed around bacteria-impregnated paper discs on agar plates was due to some product resulting from the bacterial hydrolysis of the agar. Eppley and MaciasR (1963) reported that the growth of the alga Chlamydomonas mundana Gerloff in waste stabilization lagoons was encouraged by acetate production by anaerobic bacteria. Keating (1976) discovered that the filtrates of a blue-green alga, Anabaena sp., was less inhibitory to various diatom species when a small volume of a mixed bacterial sample was inoculated into the algal cultures. According to the investigation, some of the bacteria probably metabolized the inhibitory molecules.

In another study, axenic cultures of male and female Oedogonium cardiacum failed to develop oogonia or sperm except occasionally at very low levels. The addition of a species of Corynebacterium contaminating the original culture of alga to the axenic cultures, however, increased the development of the reproductive structures. More strikingly, the addition of Pseudomonas putida to the algal cultures resulted in the growth of much longer filaments than those characteristic of either the axenic culture or the Coryne-

bacterium-contaminated alga (Machlis, 1973). Thus a bacterial-derived growth factor is evident.

A similar morphogenetic relationship was established for two marine seaweeds, Monostroma oxyspermum and Ulva lactuca (Provasoli and Pintner, 1964). Grown in the absence of other microorganisms, the two algae completely lose their typical morphology. When these axenic cultures were inoculated with a marine bacterium or a marine yeast originally associated with the seaweeds, striking effects were noted. With Monostroma, flat thallus formation was induced by these microorganisms. Culture supernatants, including autoclaved samples, were also capable of inducing this effect, as were supernatants of bacteria-free cultures of several red and brown algae.

The toxicity of bacteria against various algae has been explored to a somewhat greater degree than has the stimulation of algae by bacteria but virtually none of these studies have been in situ. Most studies have been confined to the toxigenic effects of particular bacterial species against blue-green and green algae native to fresh water. Many of these investigations involve bacterial strains in groups characterized as being cellulolytic. Daft and Steward (1971) found four bacterial isolates from fresh water which could lyse over forty strains of blue-green algae. Lysis depended upon the presence of the growing pathogen, and bacteria-free filtrates alone were insufficient

to cause lysis. Myxobacteria capable of lysing algae were also isolated by Stewart and Brown (1969) from sewage, and by Gromov et al. (1972) and Shilo (1970) from ponds. The latter two groups found evidence that a close contact between the myxobacter and an alga was necessary for lysis. In contrast, Granhall and Berg (1972) found that culture fluids from two Cellvibrio strains contained heat-resistant, low molecular weight (1,000-10,000) substances with antibiotic-like effects on blue-green algae. Cell wall synthesis was apparently impaired, according to the investigators.

Cell wall vulnerability was the subject of a related study by Gunnison and Alexander (1975a). This group found that some algae were more susceptible than others to breakdown when inoculated with pond water, and that this was directly correlated with the relative biodegradability of specific components of the algal cell walls. A follow-up investigation (Gunnison and Alexander, 1975b) disclosed that cellulase and polygalacturonase but not lysozyme, chitinase, beta-glucuronidase, hemicellulase, lipase, and pronase extensively degraded the cell walls of two green algae. The intact cells were converted to spheroplasts. The investigators concluded that susceptibility of these two algal species to microbial degradation involved an attack on the cellulose in their walls. In contrast, they found that a blue-green alga (Cylindrospermum sp.) was acted upon, and in some cases converted to spheroplasts, by lysozyme but not by other en-

zymes.

Besides this evidence that cellulolytic factors may be involved in the suppression of algal growth, other growth-inhibiting factors have been demonstrated. Shiaris and Morrison (1976), for example, isolated from a sewage oxidation lagoon a strain of Pseudomonas fluorescens capable of inhibiting a variety of blue-green algae. The algistatic substance was an extracellular, low molecular weight, non-protein compound. Production of this metabolite required a nutrient factor in the medium that was provided by either a complex substrate (beef extract - peptone) or growing blue-green algal cultures. In another investigation (Berland et al., 1972b), the filtrate from a sparse culture of Pseudomonas aeruginosa enhanced growth of the marine chlorophyte Tetraselmis striata, while the filtrate from denser populations strongly inhibited the alga. This bacterium as well as Pseudomonas marinoglutinose and others, inhibited a number of other marine algae. Pseudomonas was also implicated in the decline of Chlamydomonas and Skeletonema in liquid cultures incubated in the dark (Mitchell, 1971).

Other species of bacteria have also been implicated in algal inhibition. A strain of Bacillus brevis isolated from a sewage oxidation pond was found to lyse a number of blue-green algae (Reim et al., 1974). The lytic factor was found in culture filtrates of stationary phase bacterial cultures. The bacterium Bdellovibrio bacteriovorus, which

is commonly known to attack other bacteria (e.g., see Venosa, 1975), has also been reported to lyse the alga Phormidium luridum var. olivacea Boresch (Burnham et al., 1976). A heat-resistant lytic factor is involved. Algae are also subject to attack by viruses (Safferman and Morris, 1964; Paden et al., 1967). A phage associated with the bacterium Caulobacter vibrioides apparently was responsible for the lysis of Chlorella pyrenoidosa (Zavarzina, 1964).

That algae are susceptible to antibiotics is well documented (Krauss, 1962). They are also vulnerable to other chemicals found in nature. Using paper discs on agar plates, McGrattan et al. (1976) found that a number of fatty and related acids could inhibit the growth of Chlorella pyrenoidosa Chick. In general, unsaturated acids showed greater inhibition than the corresponding saturated acids. Of the saturated acids, the greatest growth-inhibiting activity was observed in the C_7-C_{12} range. This group also reported that acrylic acid could inhibit the alga at a 0.001M concentration. The toxicity of acrylic acid to strains of Chlorella pyrenoidosa and Chlorella vulgaris was also indicated by Sullivan and Ikawa (1972). Aflatoxin also inhibited several of the Chlorella strains. Apparently the mechanism of toxicity does not involve a lowering of pH. Chlorella vulgaris in liquid cultures at pH values ranging from 3.6 to 7.6 did not reveal any differences in the rate of photosynthesis (Nielsen and Willemoes, 1966).

In addition to toxins and other chemicals released by bacteria, suppression of algal growth may also be due to an inability by the algae to favorably compete with the bacteria for nutrients. Competition for nutrients has been examined by a number of investigators, and in virtually all cases the algae cannot compete effectively with the bacteria. These investigators have concluded that algae generally have too low an affinity for most organic substances to compete effectively under natural conditions (Neilson and Lewin, 1974; Munro and Brock, 1968; Coughlan, 1977). Moreover, there is evidence that bacteria can also compete successfully with algae for at least some inorganic substances (Rhee, 1972). Yet there may be a sufficient concentration of nutrients in the water or in microzones surrounding an algal cell so that in some cases algal heterotrophy may represent an important phenomenon (Droop, 1957; Lange, 1976; Eppley and MaciasR, 1963). Lange (1976) found evidence that suggests that the algal sheath around the cells of many algae may provide a microenvironment where essential nutrients, present in very low amounts in the surrounding water, are concentrated and made available to the cell. There is also some evidence that algae can successfully compete with bacteria for organic nutrients under low light intensities (Jolly et al., 1975; Wetzel, 1975; Maeda and Ichimura, 1973). The fact that algal heterotrophy and photoassimilation are so widespread indicates some competitive advantage in these processes.

Heterotrophic Nitrification

One of the more important transformations in nature is that of nitrification, the biological oxidation of reduced organic or inorganic nitrogen compounds. Classically, only chemoautotrophs--especially Nitrosomonas and Nitrobacter--have been implicated in this process. More recent investigations, however, have shown that numerous heterotrophic organisms can also nitrify--at least in vitro. Verstraete (1975) has recently published a review of heterotrophic nitrification.

Almost all of the work performed to date has involved the isolation and characterization of pure cultures of heterotrophic nitrifiers. These organisms were mainly isolated from soil samples and incubated with various forms of reduced nitrogen compounds--organic and inorganic--in liquid media. After various periods of incubation, the media were analyzed for the presence of nitrite and perhaps other forms of nitrogen. These studies have shown that large numbers of diverse organisms--including bacteria, fungi, and actinomycetes--can oxidize a large number of nitrogenous substrates. In constrast to the autotrophic nitrifiers which can nitrify over 2000 µg/ml of nitrogen in a liquid medium (Alexander, 1977), the heterotrophic nitrifiers generate only a small amount of nitrite, and seldom produce nitrate. Most species produced only a few µg/ml of nitrite-N at the most, although

an exceptional strain of Pseudomonas aeruginosa generated 284 µg/ml after 51 hours of incubation (Obaton et al., 1968). Nitrate levels generated were somewhat higher, with Arthrobacter producing about 4 µg/ml of nitrate-N (Gunner, 1963; Verstraete and Alexander, 1972a) and Aspergillus flavus ranging from a fraction of a µg/ml to 100 µg/ml (Hirsch et al., 1961), depending on the substrate.

Some of the heterotrophic organisms capable of nitrification, along with the substrate oxidized and products generated, are listed in Table 1. Among the identified products of heterotrophic nitrification are hydroxylamine (Gunner, 1963; Verstraete and Alexander, 1972a), bound hydroxylamine (Marshall and Alexander, 1962), amino-oxides (Cornforth and James, 1956), nitrite (Quastel et al., 1950), nitrate (Schmidt, 1954), hydroxamic acid (Verstraete and Alexander, 1972a), and primary nitro compounds (Bush et al., 1951). Nitrite and nitrate levels were found to be dependent upon the organism and the substrate (Doxtader and Alexander, 1966; Eylar and Schmidt, 1959).

As is suggested by Table 1, a great many nitrogen compounds can support heterotrophic nitrification. The presence of these nitrogen compounds in nature in terms of structure, availability, and concentration, however, is largely speculative. In one case, the existence of oximes in the soil has been debated, with Jensen (1951) considering their presence likely and Alexander (1965) disputing

TABLE 1

PARTIAL LIST OF KNOWN HETEROTROPHIC NITRIFIERS

MICROORGANISM	NITROGEN SUBSTRATE	PRODUCTS DETECTED	REFERENCE
Achromobacter sp.	PO	NI	119,120
Agrobacterium sp.	PO	NI	68
Alcaligenes sp.	PO	NI	68
	OX	NI	68
Arthrobacter globiformis	ammonium	HA,NI,NA	59
Arthrobacter sp.	ammonium	HA,NI,NA NO,NS	159,160
	acetamide	HA	
	glutamine	HA	
	glutamate	HA	
	HA	NI,NS	
	HXAA	HA,NS	
	NO	NI,NA	
Aspergillus flavus	ammonium	BHA,NI,NA	1,94
	amino acids	BHA,NI,NA	94
	NO	NI	94
Aspergillus wentii	ammonium	HA,NI,NA	2
Corynebacterium sp.	PO	NI	119,120
Fusarium sp.	PO	NI	37
Microbacterium sp.	HA	NI	30
Nocardia corallina	PO	NI	68
Proteus sp.	HA	NI	30
Pseudomonas aeruginosa	OX	NI,NO	9,112
	NO	NI	9,112
	HA	NI	9
Pseudomonas desmolyticum	urea	NA	*
	semicarbazide	NA	*
Pseudomonas sp.	HA	NI	30
Streptomyces alanosinicus	ammonium	NS	103
Streptomyces nitrificans	urethane	NI	128

*Unpublished Ph.D. thesis by J.D. Douros (1958), as cited by Doxtader and Alexander (1966).

KEY

BHA--unspecified bound HA
HA--hydroxylamine
HXAA--hydroxamic acid
NA--nitrate
NI--nitrite
NO--nitro compound
NS--nitroso compound
OX--oximes, except PO
PO--pyruvic oxime

this view. The latter claims the widespread use of the oximes as substrates in heterotrophic nitrification studies is largely irrelevant since most of the soil nitrogen in nature is in the reduced state; the high yields from these oximes, according to Alexander, have little relationship to natural conditions.

The first comprehensive survey of the heterotrophic population in soil for the ability to nitrify was published in 1959 by Eylar and Schmidt. They tested 978 isolates from twelve actively nitrifying soils for the ability to generate nitrite or nitrate in glucose peptone broth. None of the isolates yielded significant amounts of nitrite, and only 7% of them formed nitrite-N in excess of 0.2 µg/ml. In the twelve soils studied, 26% of the bacteria, 17% of the fungi, and 27% of the actinomycetes were able to generate nitrite. Similar results for actinomycete activity was reported by Hirsch et al. (1961). Thus apparently a large number of diverse forms are capable of limited nitrite production.

In the Eylar and Schmidt study, none of the actinomycetes or bacteria could consistently generate as much as 5 µg/ml nitrate-N. In contrast, 18 fungi (3.6% of the total fungi observed) could produce nitrate, but 16 of these were identified as strains of A. flavus. The level of nitrate generated varied greatly from 0 to 90 µg/ml, depending on the strain, the substrate, and the incubation conditions.

The great nitrification capacity (at least for

heterotrophs) of A. flavus has led other investigators to study the organism more extensively (Aleem and Lees, 1964; Hirsch et al., 1961; Marshall and Alexander, 1962; Schmidt, 1954). In one investigation, over 100 µg/ml of nitrate-N was found for one strain whem ammonium was used as the sole nitrogen source in a glucose-inorganic salts medium (Hirsch et al., 1961). In another study, cell-free extracts of A. flavus (as well as A. wenti and Penicillium aterovenatum) could oxidize ammonium to nitrate via hydroxylamine and nitrite (Aleem and Lees, 1964). It is noteworthy that this fungal species is widely distributed in soils.

The first heterotrophic bacterium conclusively observed to effect nitrification to the nitrate level was Arthrobacter globiformis (Gunner, 1963). About 4.5 µg/ml nitrate-N was detected after five days of incubation in a medium amended with ammonium as the sole nitrogen source. More recently, Verstraete and Alexander (1972a) investigated an Arthrobacter species isolated from sewage and found it also capable of nitrifying to the nitrate level. Arthrobacter, like A. flavus, is widespread in soils, accounting for between 5% and 60% of the total colonies isolated (Alexander, 1977).

In contrast to the numerous reports involving pure culture studies, there have only been a few articles published where field investigations have been undertaken to assess the role of heterotrophic nitrifiers in nature. In

one such investigation, by Verstraete and Alexander (1973), samples of river water, lake water, raw sewage, and loam soil were collected and amended with ammonium and acetate. Two of four sewage samples, one of four river water samples, one of four lake samples, and three of four soil suspensions displayed a pattern of nitrification resembling that of an Arthrobacter species the investigators had previously isolated and characterized. Heterotrophic nitrification was not, however, observed in soil samples either adjusted to field capacity or perfused. The authors concluded that the contribution of heterotrophic nitrifiers to the overall nitrification process was probably small, except under appropriate conditions of pH (slight alkalinity preferred) and carbon and nitrogen supply.

The ecological significance of heterotrophic nitrification has also been examined using specific inhibitors of the autotrophic nitrifiers. One of these--2-chloro-6-(trichloromethyl)-pyridine(N-Serve)--strongly inhibits these organisms, presumably leaving the heterotrophs unaffected (Shattuck and Alexander, 1963). In one in vitro experiment using ammonium fertilizer amended with N-Serve (or other inhibitors of autotrophic nitrification), strong inhibition (over 90%) was found for long periods of time at very low inhibitor concentrations (Goring, 1962). Similarly, Bundy and Bremner (1973) found that 10 ppm N-Serve mixed with soil and 200 ppm ammonium-N inhibited nitrification by 87-89%

after 28 days at a temperature of 15°C. The effectiveness of the inhibitors studied was markedly affected by soil type and soil temperature. Strong inhibition of nitrification was also noted for lake samples amended with N-Serve, but recovery was somewhat quicker (Chen et al., 1972).

Information regarding the biochemical mechanism of heterotrophic nitrification is scant. Most of the pure culture studies described previously, however, did include an analysis of the products generated with different substrates. Collectively, they disclose that amines and amides can be oxidized to hydroxylamine, hydroxamic acids, and nitrite, while hydroxamic acid as a substrate can result in the production of nitrite, hydroxylamine, and 1-nitrosoethanol. Nitrite can be produced from oximes, organic nitro compounds, urethane, nitrophenols, and other substrates.

Besides the demonstration of the presence or absence of a particular intermediate, enzyme isolation and characterization is also invaluable for elucidating the oxidation sequence of nitrogen compounds. A number of investigators have examined enzymes from actively nitrifying strains and most have been characterized as peroxidases or catalases (De Groot and Lichtenstein, 1960; Marshall and Alexander, 1962; Hirsch et al., 1961; Verstraete and Alexander, 1972b). According to Alexander (1965), peroxidases are known to catalyze a number of reactions involving nitrogen metabolism, and these include the oxidation of hydroxylamine,

nitrite, and nitroalkanes. Hirsch et al. (1961) found that the optimum pH for peroxidase formation in A. flavus (6.5-7.0) was lower than that for peroxidase activity (7.6-8.3). By contrast, nitrate and nitrite formation from ammonium was most pronounced at pH 7.0-7.5. In another experiment, Verstraete and Alexander (1972b) reported that when an Arthrobacter species was grown in a medium amended with hydroxylamine, the peroxidase and catalase activity was quite low. The oxidation of the hydroxylamine, however, was quite slow, and the presence of one or both of the enzymes could not be discounted.

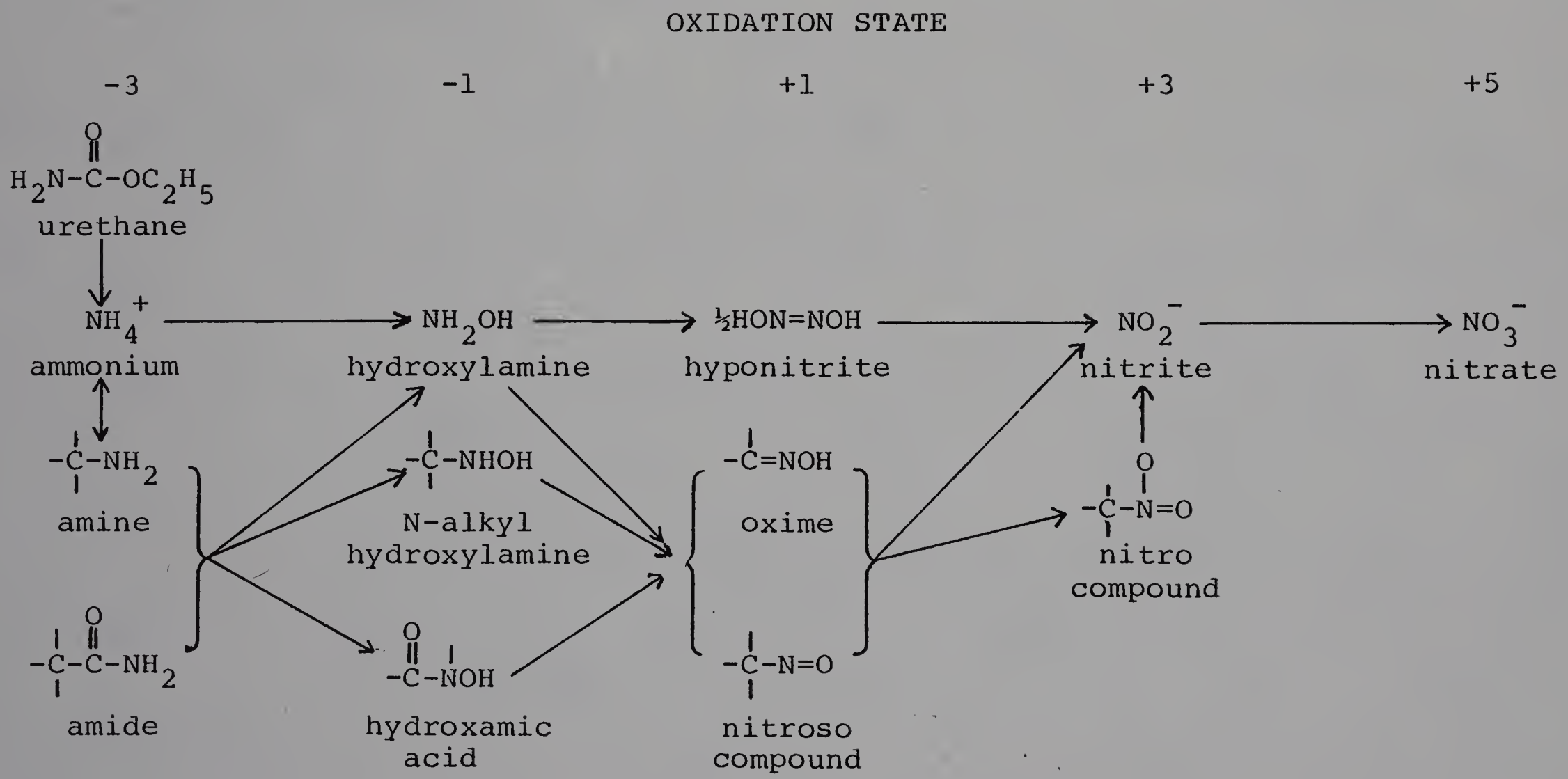
Besides peroxidase and catalase, several other enzymes associated with heterotrophic nitrification have been characterized. In one investigation, Obaton et al. (1968) found two distinct enzymes were responsible for the formation of nitrite in P. aeruginosa. The enzyme catalyzing the generation of nitrite from nitroethane was constitutive, while the enzyme catalyzing the release of nitrite from oxime solutions was soluble and inducible. Nitrite was produced more rapidly from nitroethane than from the oximes in this bacterium. In another study, Marshall and Alexander (1962) found evidence for the presence of nitrate reductase in cultures of A. flavus. Other enzymes associated with heterotrophic nitrification are pyruvic oxime oxidase (Quastel et al., 1950) and cytochrome C reductases (Aleem et al., 1964).

The foregoing information suggests that both inorganic and organic compounds may be involved in heterotrophic nitrification. A particular oxidative pathway may be strictly inorganic, completely organic, or a combination of the two. In contrast to this, autotrophic nitrification is thought to occur by a strictly inorganic sequence (Alexander, 1977). The oxidation sequence of nitrogen, whatever the substrate, is posulated to occur as a stepwise reaction, one intermediate or product having an oxidation state two above its immediate predecessor (Alexander et al., 1960). A composite set of sequences is represented in Figure 1, based on the results of a number of investigators. It is very likely that a heterotrophic nitrifier will oxidize nitrogen along at least a portion of one or more of these sequences.

Metabolic factors determining the specific pathway of nitrogen oxidation are not known for certain. It has been demonstrated that, at least for Arthrobacter, hydroxamic acids accumulate only in iron-deficient media (Verstraete and Alexander, 1972a). Perhaps the special affinity of the hydroxamate anion for ferric ions plays a role in facilitating growth in this environment (Neilands, 1967). It has also been suggested that free hydroxylamine is favored by a low pH, but binds to a carbon skeleton more readily at a neutral pH (Marshall and Alexander, 1962).

While the mechanisms for pathway preference are not well understood, it has been demonstrated that heterotrophic

Figure 1. Possible sequences in heterotrophic nitrification (modified from Alexander et al., 1960).



nitrifiers are not obligate oxidizers of reduced nitrogen compounds. A number of strains of A. flavus, for example, can grow well on media amended with ammonium or urea, but do not produce either nitrite or nitrate as they can on other types of media (Eylar and Schmidt, 1959).

MATERIALS AND METHODS

Organisms. A number of bacteria were isolated by standard dilution plate techniques or enrichment techniques from the littoral zone of Quabbin Reservoir and Pontoosuc Lake in central Massachusetts. Three of the bacteria most frequently employed in this investigation were identified as Arthrobacter sp. (hereafter referred to as Arthrobacter sp. Q1), Flavobacterium sp., and Nocardia sp. Their morphological and biochemical characteristics are listed in Appendix D. In addition, several bacterial species were obtained from local culture collections. All algae were obtained from the Carolina Biological Supply Co. They are identified below, along with their Indiana University Culture Collection number.

Anacystis nidulans	625
Ankistrodesmus falcatus	101
Chlamydomonas moewusii	97
Chlorella vulgaris	398
Chlorococcum hypnosporum	119
Closterium acerosum	1076
Oscillatoria tenius	1566
Scenedesmus quadricauda	614

Chlorella was purified by repeated streakings on Plate Count Agar (PCA) plates. Chlorococcum, Chlamydomonas, and Ankistrodesmus were purified by repeated streakings on plates containing Bold's Basal Medium (BBM) plus 1.5% agar.

Media. Media used in this investigation are presented below.

1. Bold's Basal Medium. This is a mineral salts liquid medium often used for algal growth. It is more fully described in Appendix C.

2. Plate Count Agar (Difco)

3. m-Plate Count Broth (Difco)

4. Nutrient Agar (BBL)

5. Nutrient Broth (Difco)

6. Special Agar--Noble (Difco)

7. Ammonium-acetate medium. This is a liquid medium sometimes used to determine an organism's ability to nitrify. It is more fully described in Appendix C.

Water used for all media was distilled and deionized (Milli-Q distilled water system, Millipore Corp.). All media were sterilized in an autoclave.

Maintenance of cultures. Bacteria were transferred to fresh Plate Count Agar (PCA) slants monthly, incubated at room temperature, and refrigerated. Axenic algal cultures were refrigerated in the dark on slants consisting of BBM plus 1.5% agar. To insure a constant source of active algae, inoculations from the slants to acid-clean 300 ml Erlenmeyer flasks containing 100 ml BBM were accomplished as required. These flasks were incubated at room temperature on a rotary shaker under two 40-watt cool-white fluorescent lights.

Preparation of organisms and their filtrates for experiments. Bacteria were cultured in m-Plate Count Broth overnight.

Cultures were centrifuged and washed twice with BBM and then resuspended in a tube of BBM. Optical density was measured at an appropriate dilution at 680 nm. It was necessary to wait several hours before determining optical density to permit the dissolution of bacterial aggregates. Aliquots of algae were aseptically removed from the maintenance flasks as needed and centrifuged and washed twice with BBM. Chlorella cells used in pour plate experiments were not washed. The optical density of algal cells was also measured at 680 nm. Bacterial and algal filtrates were obtained by centrifugation of the culture. These were filter sterilized using a Millipore "Sterifil" filter system or a Millipore "Swinnex" filter holder with a Millipore 0.22 μ membrane (type GS). Bacterial and algal cells were examined microscopically for purity before use. A small aliquot of all filter-sterilized filtrates was transferred to a tube of m-Plate Count Broth and also streaked onto a PCA plate to check for bacterial contamination.

Growth studies in liquid media. For all interactions between live algae and live bacteria cells, the following protocol was used. Acid-cleaned 300 m. Erlenmeyer flasks containing 100 ml sterile BBM were inoculated with bacteria, algae or both. The inoculum usually consisted of approximately 10^6 cells. All flasks were shaken by a rotary shaker at room temperature under continuous cool-white fluorescent lighting

(two 40-watt lamps). Aliquots were aseptically drawn as needed for bacterial and/or algal counts. A similar procedure was employed when algal filtrates were used instead of BBM, except that cultures were incubated in the dark. For bacterial counts, aliquots were serially diluted as necessary in tubes containing BBM, and pour plates were prepared using PCA and plastic petri dishes. Where three bacteria distinguished by color were grown together in media, seeded plates were substituted for pour plates in enumerating bacteria because of poor color development with the latter method. Plates were incubated for five days in the dark and counted with a Quebec colony counter. Algae were counted using a hemacytometer. Chlamydomonas cells, which are motile, were killed with a drop of formaldehyde just before counting.

Preparation of Chlorella-inoculated plates. Approximately 10^4 cells of an axenic Chlorella culture growing in BBM were pipetted to each plastic petri dish. Pour plates were then prepared by the addition of 20 ml of cooled PCA per plate either from an Erlenmeyer flask or, when greater precision in volume was necessary, from test tubes containing measured amounts of PCA. When the addition of chemicals was necessary, the filter-sterilized chemical sample was carefully pipetted into the petri dish as far as possible from the Chlorella sample. Plates were then quickly poured and thoroughly swirled.

All Chlorella-inoculated plates were incubated in the dark at room temperature. Algal colonies became visually evident within three to four days. When a bacterial streak on a plate was required, a single streak using a loop was applied across the center of a one-day old Chlorella-inoculated plate, and the plate reincubated. One-day old plates were used for all bacterial streaks, agar well tests, and tests involving treatments applied to glass penicylinders. This was done to permit algal adaptation to the medium and to reduce surface moisture effects.

Preparation of agar for chemical tests. A total of 4g agar medium, taken equally from three different plates, was used in all cases. Agar was sliced from the plates and transferred to a tissue grinder (glass tube and glass pestle). Deionized, distilled water was then added, and the agar macerated. This slurry was transferred to a test tube. Water was again added to the tissue grinder to extract residual agar, and the resulting mixture was added to the original slurry. A total of 10 ml water was used in grinding. The test tube containing the agar slurry was vortexed thoroughly, and the sample subjected to immediate chemical testing.

Chemical tests. Hydroxylamine was measured by the 8-hydroxyquinoline method of Magee and Burris (1954). Nitrite was determined by the sulfanilimide procedure described in Standard Methods (1975). After color development, the agar sam-

ples were centrifuged and the optical density of the clarified supernatant was determined with a Bausch and Lomb Spectronic 20 colorimeter. Controls indicated that no significant amounts of hydroxylamine or nitrite adhered to the centrifuge-formed pellet.

The following correction factor for dilution was applied to concentrations obtained by the above procedures:

$$\text{Concentration } (\mu g/\text{ml agar}) = \frac{\text{wt. of agar + vol. water added}}{\text{wt. of agar}} \times \text{measured concentration}$$

(note: it was found that 1 ml agar weighs 1g).

Chlorophyll a determination. Chlorophyll a determinations were performed by a modification of the trichromatic method (Standard Methods, 1975). Four grams of Chlorella-containing agar from three plates were transferred to a tissue grinder and 6-8 ml 90% acetone added. The agar was macerated and poured into a screw-cap test tube. Residual agar in the grinder was macerated with a quantity of 90% acetone sufficient to bring the total level of acetone solution to 10 ml and this was added to the tube. Tubes were steeped overnight at 4°C in the dark and then clarified by centrifugation. The volume of the clarified extract was measured and the optical density determined at wavelengths of 663, 645, and 630 nm. The following formula was used to determine chlorophyll a levels:

$$\text{Chl } \underline{a} \text{ } (\mu g/ml) = 11.64D_{663} - 2.16D_{645} - 0.10D_{630}$$

The following correction term for dilution was applied to the above:

$$\text{Chl } \underline{a} \text{ } (\mu g/ml \text{ agar}) = \frac{\text{wt. of agar} + \text{vol. extract}}{\text{wt. of agar}} \times \text{measured Chl } \underline{a}$$

Occasionally after centrifucation of a sample containing heavy Chlorella growth, the agar pellet was observed to possess a green tint. To assess the impact on optical density by the loss of this green material, several pellet samples of this type were remacerated in 90% acetone and recentrifuged. Each supernatant was added to the original, previously-measured extract and the subsequent extract reduced in volume by evaporation in an air stream to that of the original. The optical densities of this sample were then determined. Differences in optical density between the original extract and the second extract were negligible.

Test for large particle inhibition of Chlorella. Several experiments were attempted to detect stable bacterial enzymes or other stable high molecular weight particles capable of inhibiting Chlorella growth in agar plates. In each study, a 300 ml Erlenmeyer flask containing 100 ml of medium was inoculated with about 10^4-10^5 Chlorella cells and about 10^7 Arthrobacter sp. Q1 cells. Chlorella was used to induce enzyme synthesis. The media used were Nutrient Broth, BBM containing 0.1% tryptone (Difco) and 0.01% yeast extract

(Difco), and unsupplemented BBM. Cultures in the first two media were incubated in the dark on a rotary shaker for 40 hours. Cultures in the unsupplemented BBM were similarly incubated for 12 days. They were then centrifuged and the supernatants subjected to ultrafiltration, using an Amicon UM2 ultrafilter. This filter permits passage of particles having a molecular weight of less than 1000. Ultrafiltration increased the concentration of larger particles by 3 for Nutrient Broth, 20 for supplemented BBM, and 15 for unsupplemented BBM. Each concentrated residue from ultrafiltration was then filter-sterilized using a 0.2 μ polycarbonated membrane filter (Nuclepore) and aseptically pipetted in a laminar flow hood into either glass penicylinders or agar wells on one-day-old Chlorella-inoculated PCA plates. Controls consisted of non-sterile residues and filter-sterilized supernatants of the ultrafiltration process. Plates were incubated in the dark, right side up. When algal plate growth became evident, plates were checked for clear areas around the agar wells or penicylinders.

Glucose test. Pour plates consisting of Chlorella cells and four different concentrations of filter-sterilized glucose were prepared and incubated in the dark. Triplicate plates were prepared for each glucose level (1%, 3%, 5%, 10%). One day later, Arthrobacter sp. Q1 was streaked along a measured distance across the center of each plate, and plates were re-

incubated. After Chlorella colonies became evident, the area of the resulting zone of inhibition around each streak was measured with a planimeter (Dietzgen, OTT type 30). The average value for each glucose level was recorded and statistically examined.

pH tolerance tests. The effect of alkaline pH values on the growth of Arthrobacter sp. Q1 was determined by inoculation of 100 ml BBM buffered at different values. Buffers were prepared according to the procedures in Methods of Enzymology (Gomori, 1955). Phosphate buffer was used for pH values 6.6 and 7.6. Boric acid/borax buffer was used for pH 8.6, and borax-potassium hydroxide buffer was used for pH 9.6. For the two higher pH values, KH_2PO_4 and K_2HPO_4 normally in BBM were omitted and replaced with an equivalent amount of K_3PO_4 (0.16 g/l PO_4^{3-}). To permit extra bacterial growth, the BBM in all flasks was supplemented with 0.1% m-Plate Count Broth. Bacterial counts were performed as previously described.

The effect of pH on Chlorella growing in PCA plates was determined by two methods. In one method, citric acid/sodium citrate buffers were prepared for pH 3.0, 4.0, 5.0, and 6.0 according to Methods of Enzymology (Gomori, 1955) and pipetted to penicylinders on Chlorella-inoculated PCA plates. The second method involved adding predetermined quantities of sterile potassium hydroxide to PCA at 45°C just before preparing pour plates containing Chlorella. The

amounts of hydroxide added were sufficient to yield agar pH values of 8.0, 8.5, and 9.2. In both methods, a lag in Chlorella growth compared to algal growth in PCA plates at pH 7.0 was considered a pH effect.

Chemicals. Hydroxylamine hydrochloride was obtained from Fisher Scientific Co. Acetaldoxime, acetone oxime, 2-butanone oxime, and 1-nitropropane were obtained from Eastman Organic Chemicals.

Electron microscopy. For observation in the electron microscope, one droplet of Arthrobacter sp. Q1 suspensions (about 10^6/ml) was applied to 200 mesh, 1% collodion-coated copper grids. After one minute, the grids were washed with distilled water and the suspensions were negatively stained with 1% ammonium molybdate (pH 7.0) for 1½ minutes. The stain was removed with filter paper and the grids were air dried. Samples were examined with a Zeiss EM 9S-2 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Effects of Algae on Bacteria

The effect of various freshwater algae on their associated bacterial flora was found in general to be stimulatory. As can be observed in Figure 2, the overall bacterial population in five of the eight monocultures of algae tested increased dramatically over a twenty day period, most of them by at least two orders of magnitude. Interestingly, two of the most stimulatory algae were blue-green algae (i.e., Oscillatoria and Anacystis). In each graph, Arthrobacter sp. Q1 in Bold's Basal Medium (BBM) is depicted as a bacterial control. This was considered appropriate even though the algal-associated bacteria may not have included this species because the Arthrobacter is a typical freshwater organism. In three cases (Chlorococcum, Chlamydomonas, and Chlorella), significant stimulation was not observed under the conditions of the experiment.

The addition of a non-indigenous bacterial strain (Serratia marcescens) to each of the nonaxenic algal cultures resulted in the rapid decrease of the Serratia population in almost every case as the algal population increases (Figure 3). This result is consistent with the well-established observation that non-native strains usually cannot effectively

Figure 2. Effects of various algae upon the growth of algal-associated bacterial populations in Bold's Basal Medium. Connected line represents this bacterial population; dashed line represents Arthrobacter sp. Q1 in Bold's Basal Medium as a control.

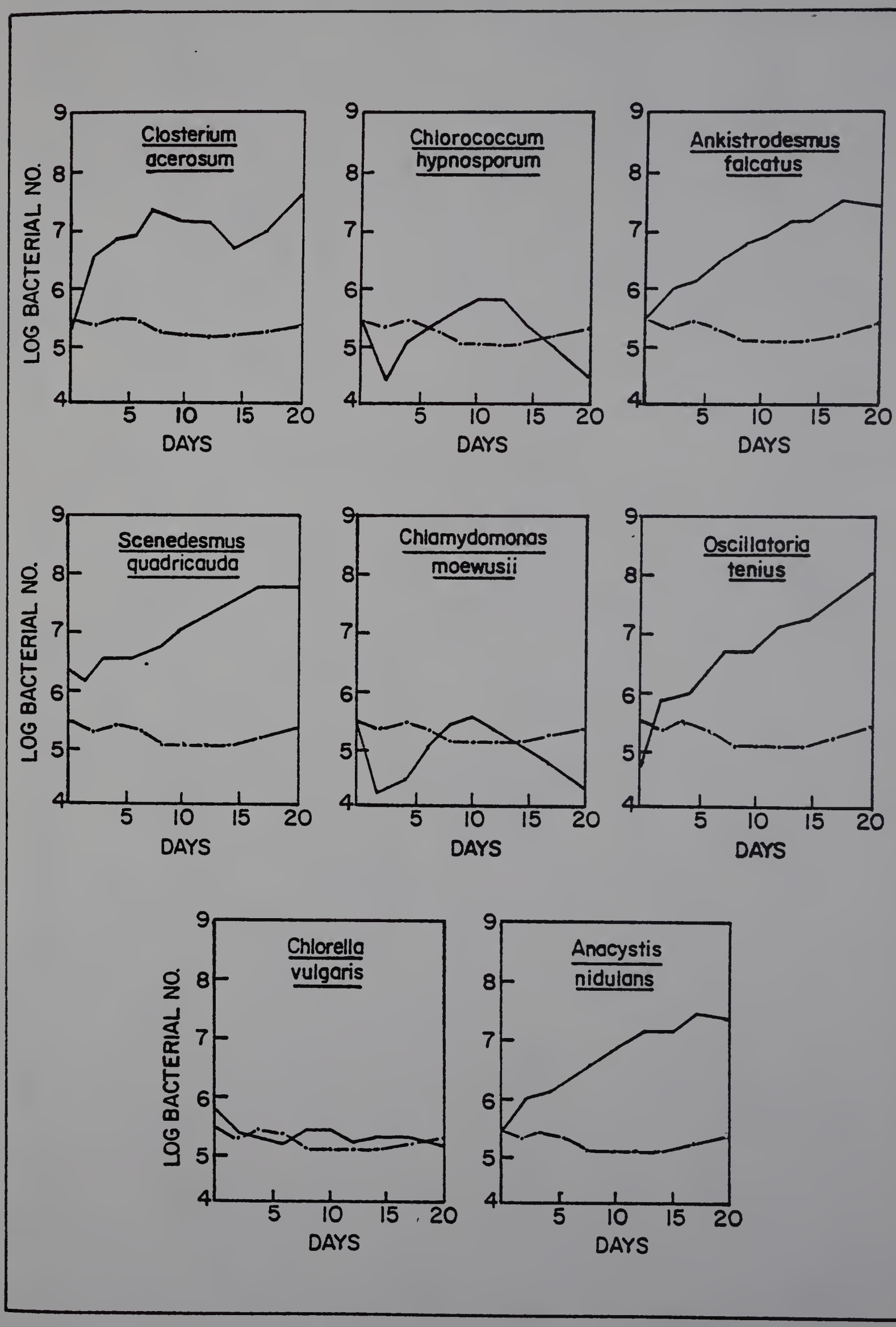
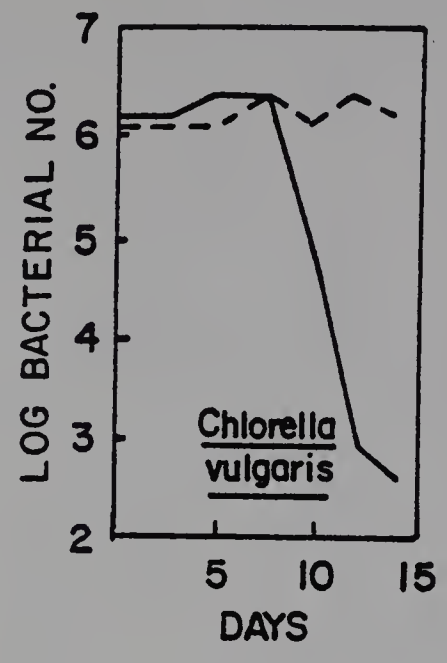
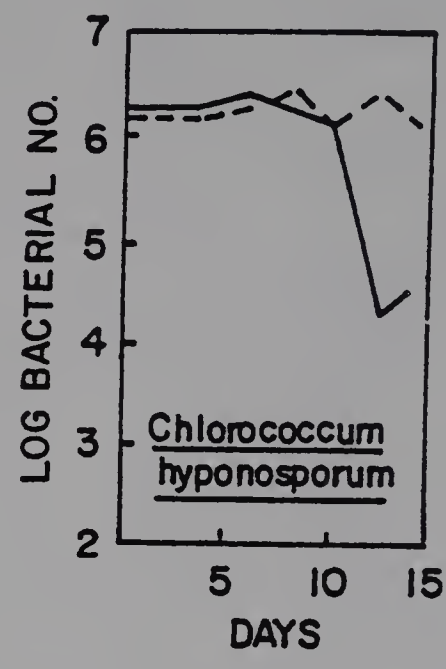
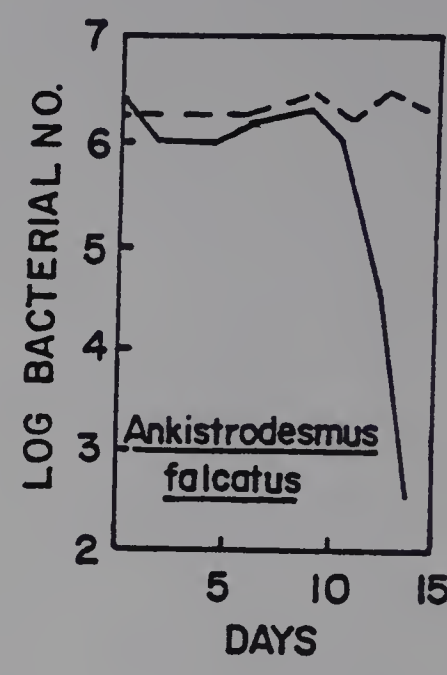
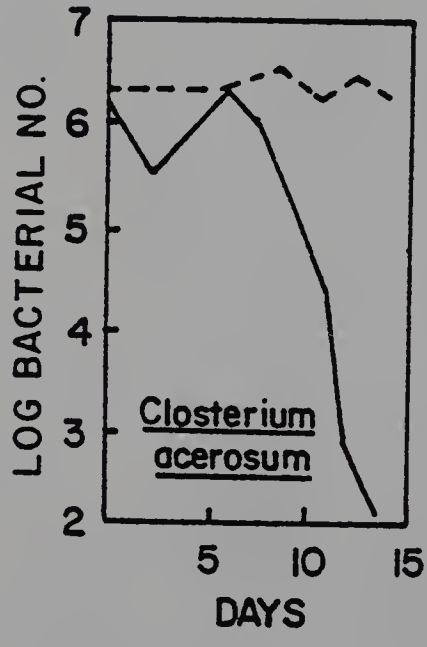

Closterium acerosum
Chlorococcum hypnosporum
Ankistrodesmus falcatus
Scenedesmus quadricauda
Chlamydomonas moewusii
Oscillatoria tenius
Chlorella vulgaris
Anacystis nidulans
LOG BACTERIAL NO.
DAYS
9
8
7
6
5
4
5
10
15
20

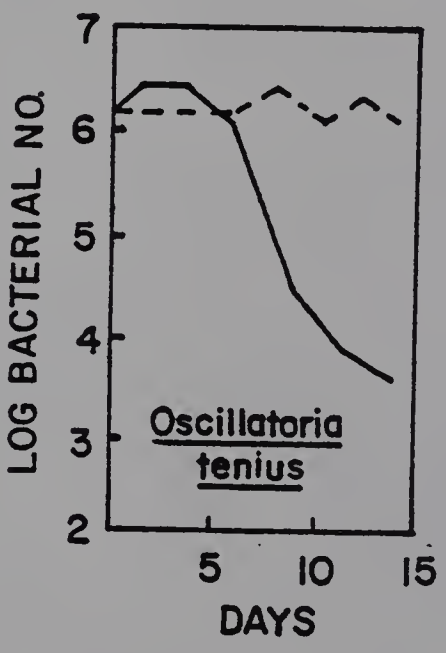
Figure 3. Effects of various algae upon the growth of a bacterial species not ordinarily associated with algae (Serratia marcescens) in Bold's Basal Medium. Connected line represents S. marcescens numbers in flasks inoculated with an alga. Dashed line represents growth of an axenic culture of S. marcescens in Bold's Basal Medium.

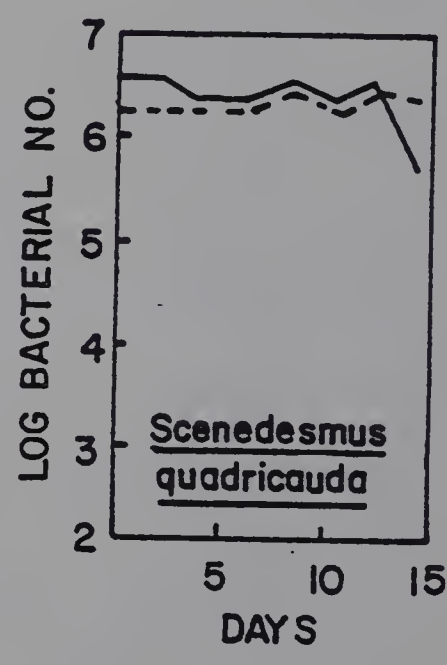

LOG BACTERIAL NO.
7
6
5
4
3
2
Chlorella vulgaris
5
10
15
DAYS

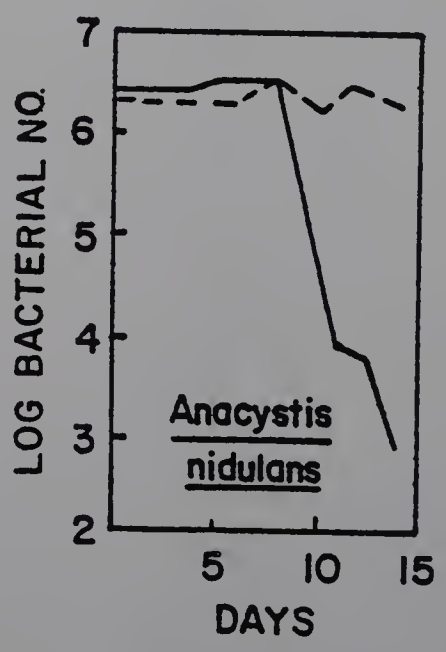

LOG BACTERIAL NO.
7
6
5
4
3
2
Chlorococcum hyponosporum
5
10
15
DAYS


LOG BACTERIAL NO.
7
6
5
4
3
2
Ankistrodesmus falcatus
5
10
15
DAYS


LOG BACTERIAL NO.
7
6
5
4
3
2
Closterium acerosum
5
10
15
DAYS


LOG BACTERIAL NO.
7
6
5
4
3
2
Oscillatoria tenius
5
10
15
DAYS


LOG BACTERIAL NO.
7
6
5
4
3
2
Scenedesmus quadricauda
5
10
15
DAYS


LOG BACTERIAL NO.
7
6
5
4
3
2
Anacystis nidulans
5
10
15
DAYS

compete with an entrenched population.

Algal-bacterial interactions which failed to stimulate the growth of the indigenous bacterial flora were examined more closely to ascertain the reason for this lack of bacterial response. Axenic cultures of Chlorella, Chlorococcum, and Chlamydomonas were prepared. All three are common in polluted waters. In addition, an axenic preparation of a stimulatory alga--Ankistrodesmus--was used for comparison studies. In most of the subsequent experiments, Arthrobacter sp. Q1 was used as the test bacterium.

The response of Arthrobacter sp. Q1 to live axenic algal cells was the first matter investigated. As is shown in Figure 4, the effect of Ankistrodesmus and Chlamydomonas on the bacterium was mildly stimulatory. Chlorococcum failed to stimulate the Arthrobacter as it had failed to stimulate the mixed indigenous flora. In contrast, Chlorella was distinctly inhibitory.

To more closely explore the reason for the failure to stimulate more strongly, the effect of algal culture filtrates was examined. In one experiment, the filter-sterilized filtrates from algal cultures were subjected to equal numbers of three freshwater bacterial isolates distinguishable by color. As shown in Figure 5, the four filtrates strongly stimulated the growth of the test bacteria. No filtrate was either more or less stimulatory than any other. The red organism (Nocardia sp.) was enhanced somewhat less

Figure 4. Respective effects of four actively growing algae on the growth of Arthrobacter sp. Q1 in Bold's Basal Medium.

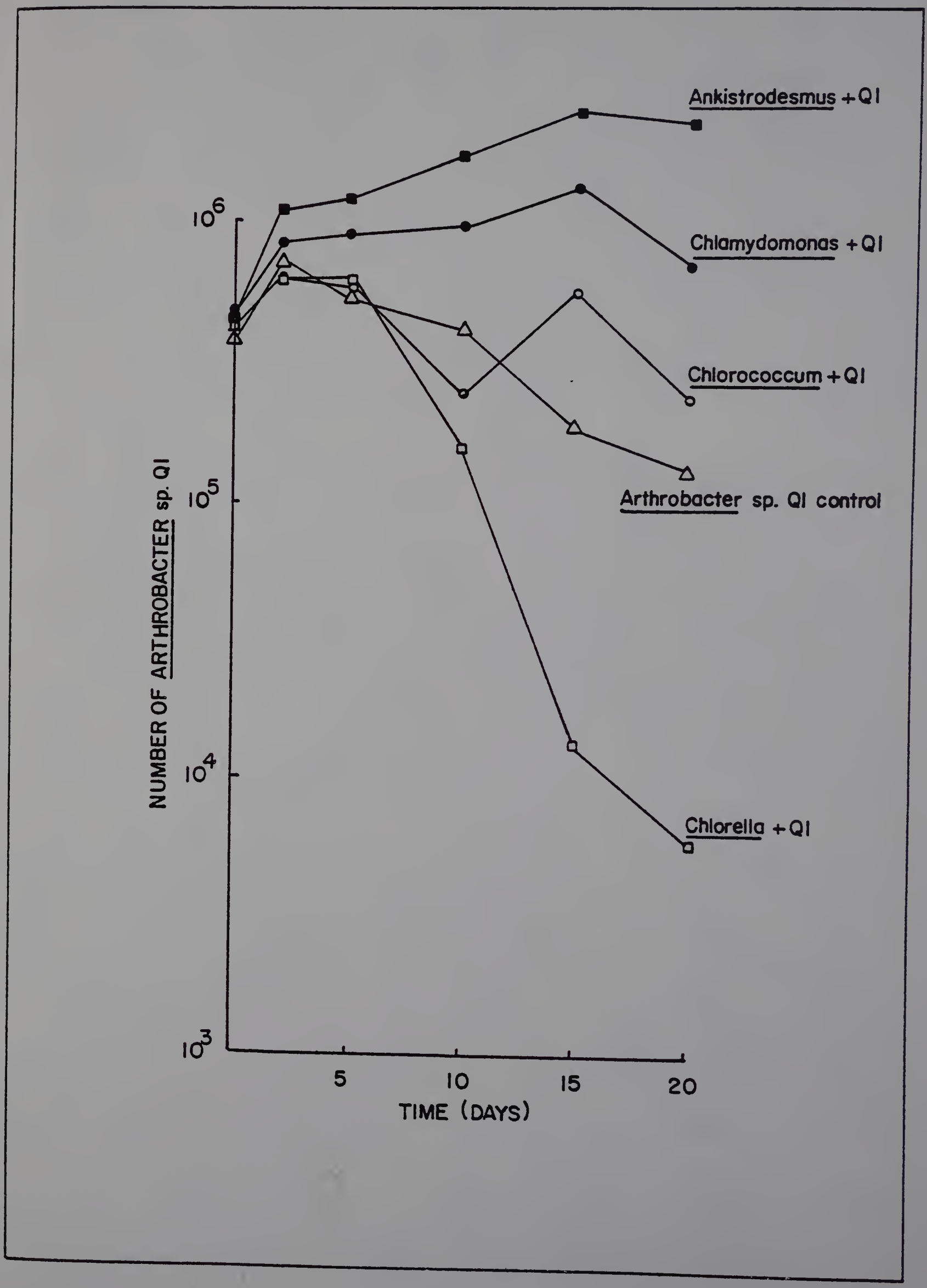
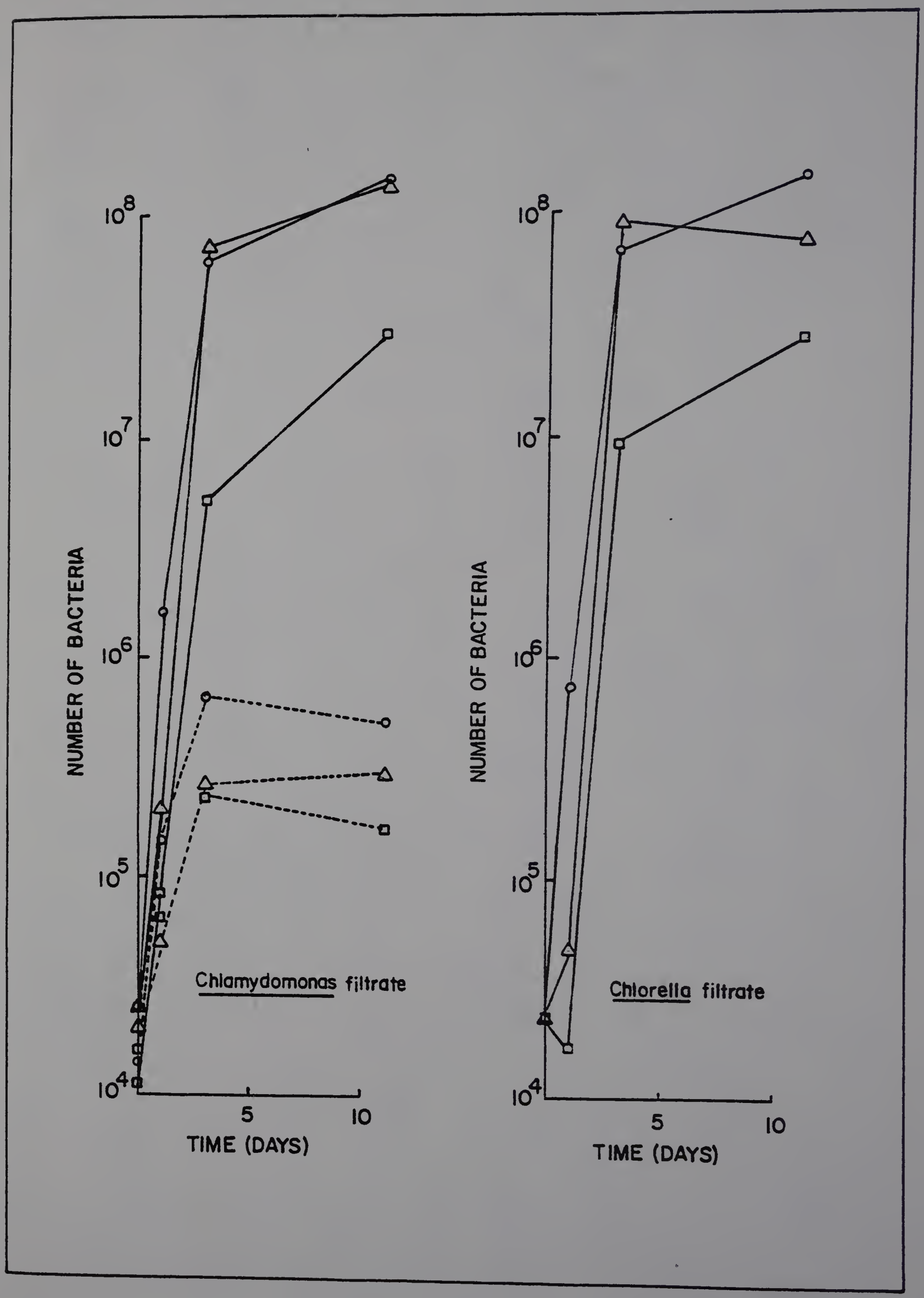
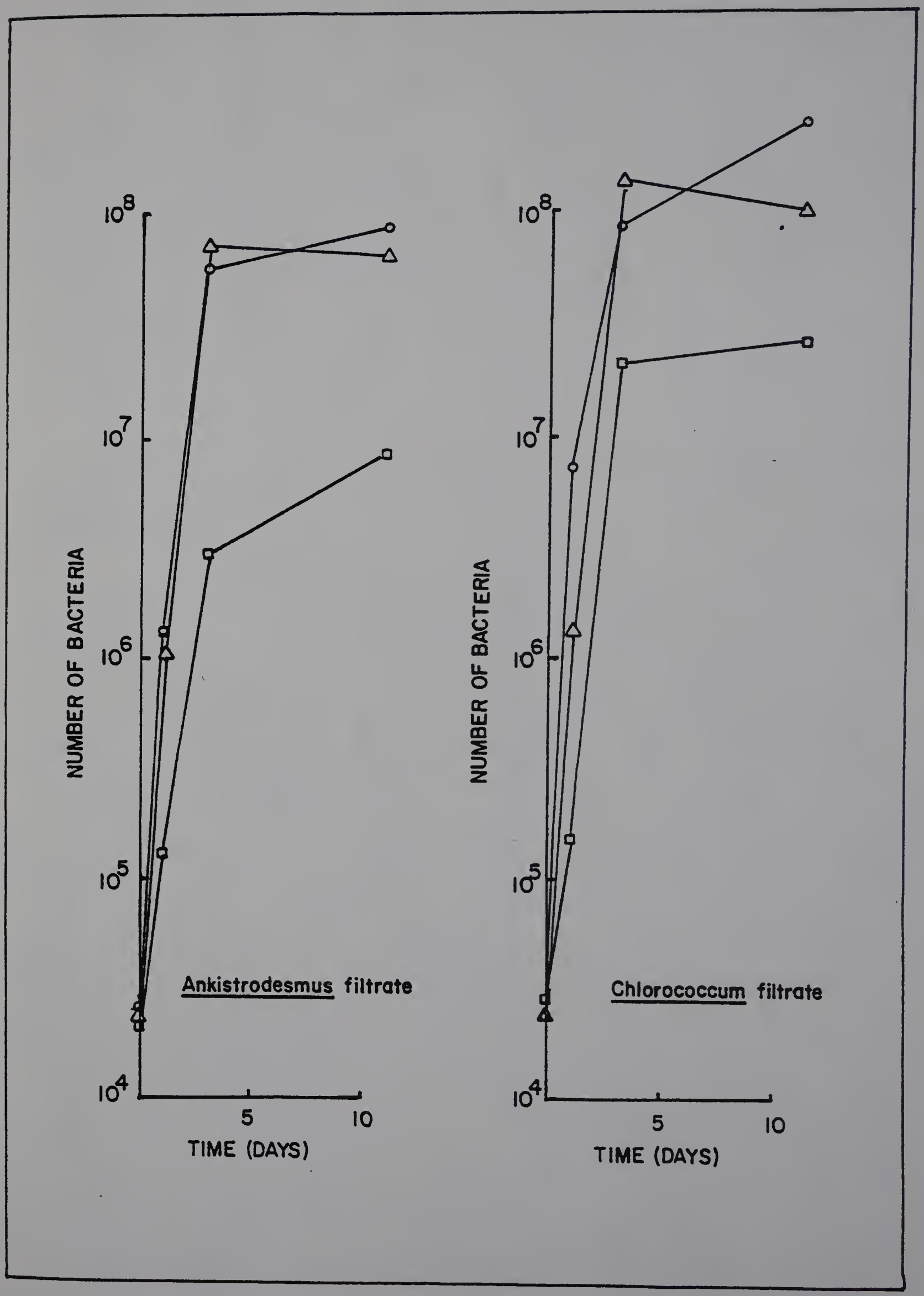

Ankistrodesmus +QI
Chlamydomonas +QI
Chlorococcum +QI
Arthrobacter sp. QI control
Chlorella +QI
NUMBER OF ARTHROBACTER sp. QI
10^6
10^5
10^4
10^3
5
10
15
20
TIME (DAYS)

Figure 5. Growth pattern of three species of lake bacteria occurring together in four filter-sterilized algal filtrates. ° represents Arthrobacter sp. Q1; Δ represents Flavobacterium sp.; □ represents Nocardia sp. Dashed lines indicate growth pattern of the three bacteria occurring together in Bold's Basal Medium without the presence of algal filtrates.


NUMBER OF BACTERIA
10^8
10^7
10^6
10^5
10^4
Chlamydomonas filtrate
5
10
TIME (DAYS)
NUMBER OF BACTERIA
10^8
10^7
10^6
10^5
10^4
Chlorella filtrate
5
10
TIME (DAYS)

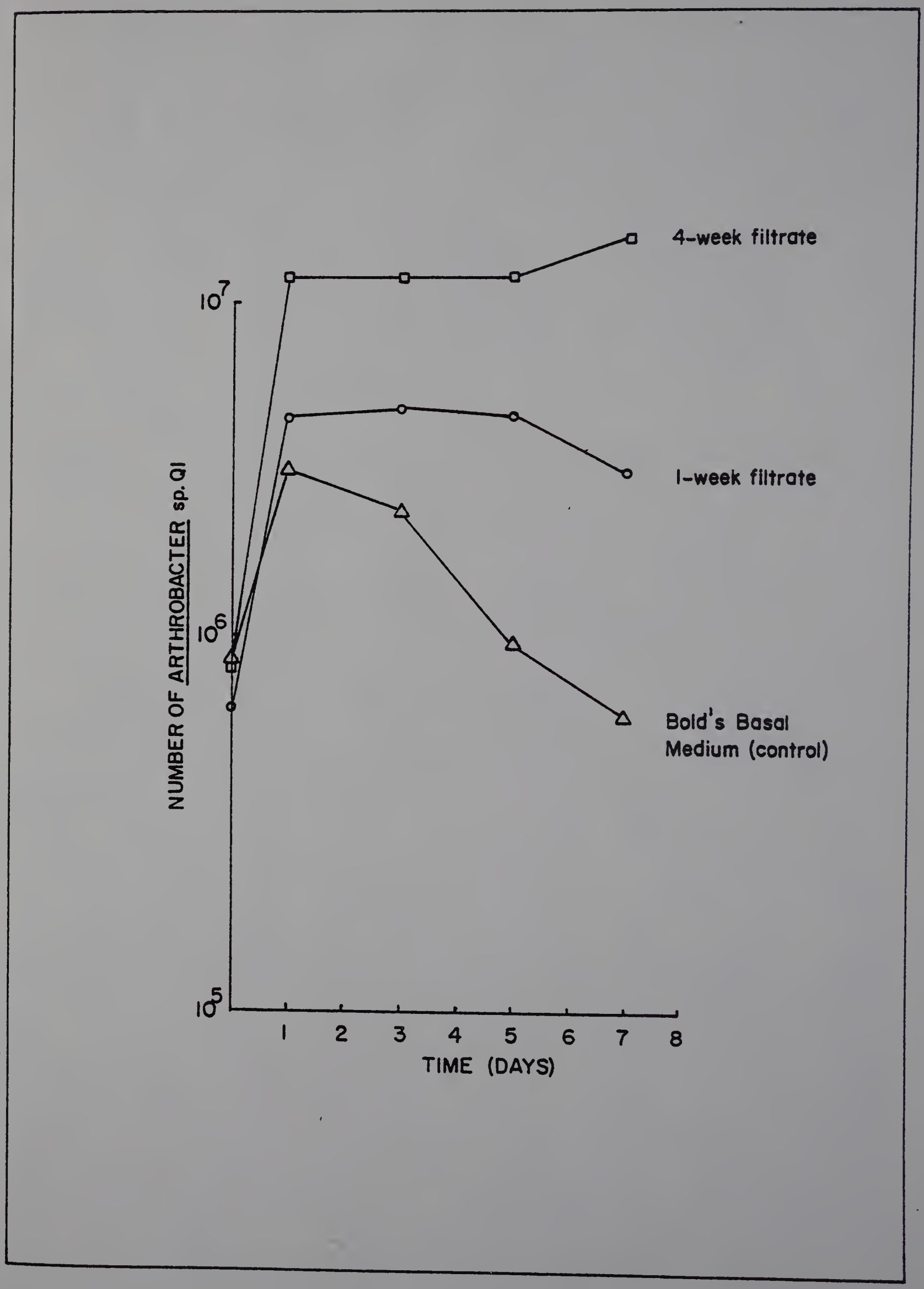

NUMBER OF BACTERIA
10^8
10^7
10^6
10^5
10^4
Ankistrodesmus filtrate
5
10
TIME (DAYS)
NUMBER OF BACTERIA
10^8
10^7
10^6
10^5
10^4
Chlorococcum filtrate
5
10
TIME (DAYS)

than the yellow (Flavobacterium) or the white (Arthrobacter sp. Q1) organism in all four filtrates. Yet even with the Nocardia sp., the population density was about two orders of magnitude above that of the mixed population control in BBM. Thus algal filtrates generally appear to strongly stimulate a wide range of bacterial populations, including Arthrobacter sp. Q1.

The age of the algal culture apparently also has an effect on the growth of Arthrobacter sp. Q1. The filtrates from a four week culture and a one week culture of Chlorococcum were compared as to their ability to stimulate the bacterium (Figure 6). The resulting bacterial population in the filtrate from the older culture was about three times the number of that in the one week filtrate. In accordance with the previous experiment, the population density of the test bacterium in the Chlorococcum filtrate was substantially above that in the BBM control. Thus the lack of stimulation of Arthrobacter sp. Q1 by actively growing algal cells of Chlorococcum, Chlorella, and Chlamydomonas would appear to be due to some stress placed upon the environment by the activities of actively growing algal cells.

Several experiments were then undertaken to determine the effects on Arthrobacter sp. Q1 growth of differences in population density between algal cells and the bacterium. When equal populations of the test bacterium were incubated with initially varying Chlorella populations, it was evident

Figure 6. Effect of age of Chlorococcum filtrate on growth of Arthrobacter sp. Q1.


4-week filtrate
1-week filtrate
Bold's Basal
Medium (control)
NUMBER OF ARTHROBACTER sp. QI
10^7
10^6
10^5
1
2
3
4
5
6
7
8
TIME (DAYS)

that the Chlorella inhibited the bacterium, and that the greater the initial Chlorella population, the greater the suppression of bacterial numbers (Figures 7 and 8). While active bacterial suppression can first be discerned during the early log phase of algal growth, the effect persists at least through the early stationary phase. In a related experiment, the plots of varying initial bacterial populations when grown with equal densities of Chlorella also differed (Figure 9). Higher initial bacterial counts resulted in greater bacterial numbers even after fifteen days. The data of these two sets of experiments suggest that the greater the ratio of Chlorella populations to Arthrobacter sp. Q1, the greater the inhibition of bacterial growth.

In contrast, algal growth is not significantly hampered by the bacterium. This can be noted in Figure 8, where two cultures containing initially equal Chlorella populations can be compared. The culture not challenged with bacterial cells (10^0 control) was not significantly different from that containing bacteria (10^0).

Contrary to population density results with Chlorella, no distinct pattern emerges when bacterial population profiles in different initial Chlamydomonas population densities are compared (Figure 10). The Chlamydomonas mildly stimulates Arthrobacter sp. Q1 growth, which is consistent with the data in Figure 4. Chlamydomonas grew much more slowly under the conditions of this experiment than did

Figure 7. Effect on bacterial growth of equal populations of Arthrobacter sp. Q1 added to varying populations of Chlorella cells in Bold's Basal Medium. A twice-washed suspension of Chlorella was prepared. The 10^{-1} curve represents the growth of a 1/10 dilution of this suspension added to an Arthrobacter population.

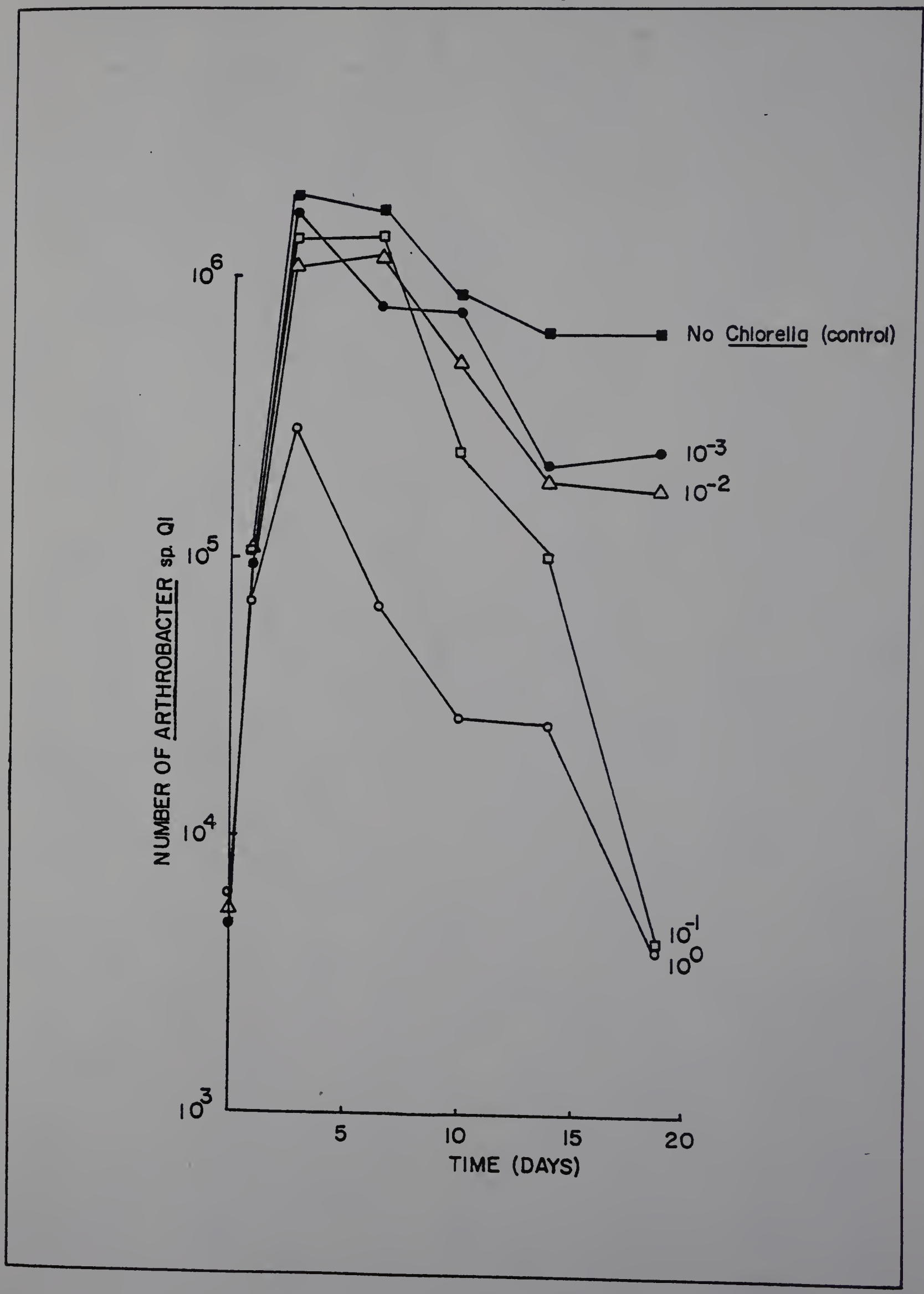

No Chlorella (control)
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{6}
10^{5}
10^{4}
10^{3}
NUMBER OF ARTHROBACTER sp. QI
5
10
15
20
TIME (DAYS)

Figure 8. Growth of Chlorella cells in experiment depicted in Figure 7. The 10^0 control represents Chlorella cells not inoculated with Arthrobacter. Because algal counts by hemacytometer are inaccurate below a population of 10,000 cells per ml, not all algal curves begin at day 0. ▲ represents 10^0 control; ° represents 10^0 dilution; □ represents 10^{-1} dilution; Δ represents 10^{-2} dilution; • represents 10^{-3} dilution of the alga.

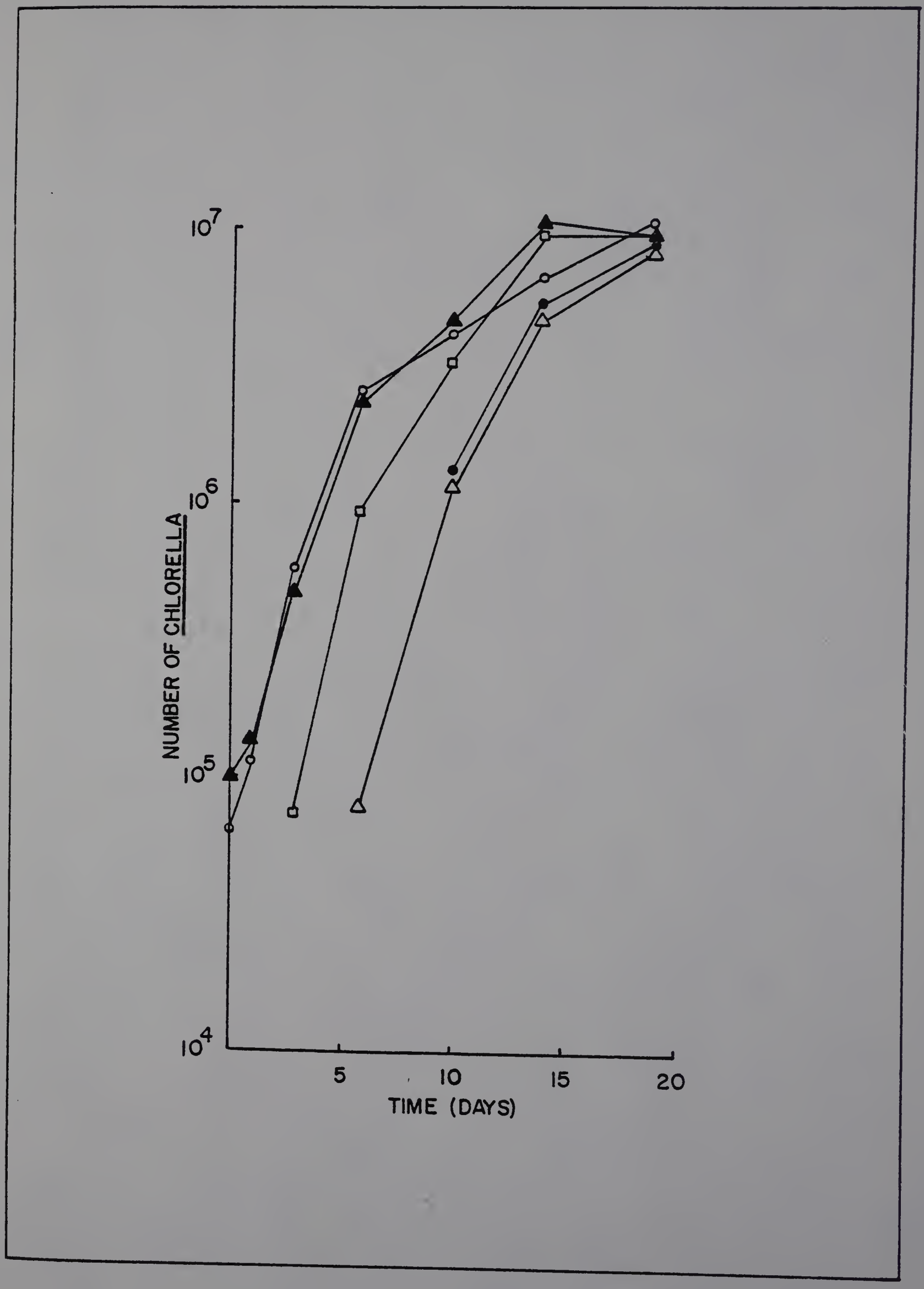

NUMBER OF CHLORELLA
10^7
10^6
10^5
10^4
5
10
15
20
TIME (DAYS)

Figure 9. Effect on bacterial growth of a constant number of Chlorella cells added to different population densities of Arthrobacter sp. Q1. A twice washed suspension of Arthrobacter was prepared. The 10^{-1} graph represents the growth of a 1/10 dilution of this suspension added to a Chlorella population.

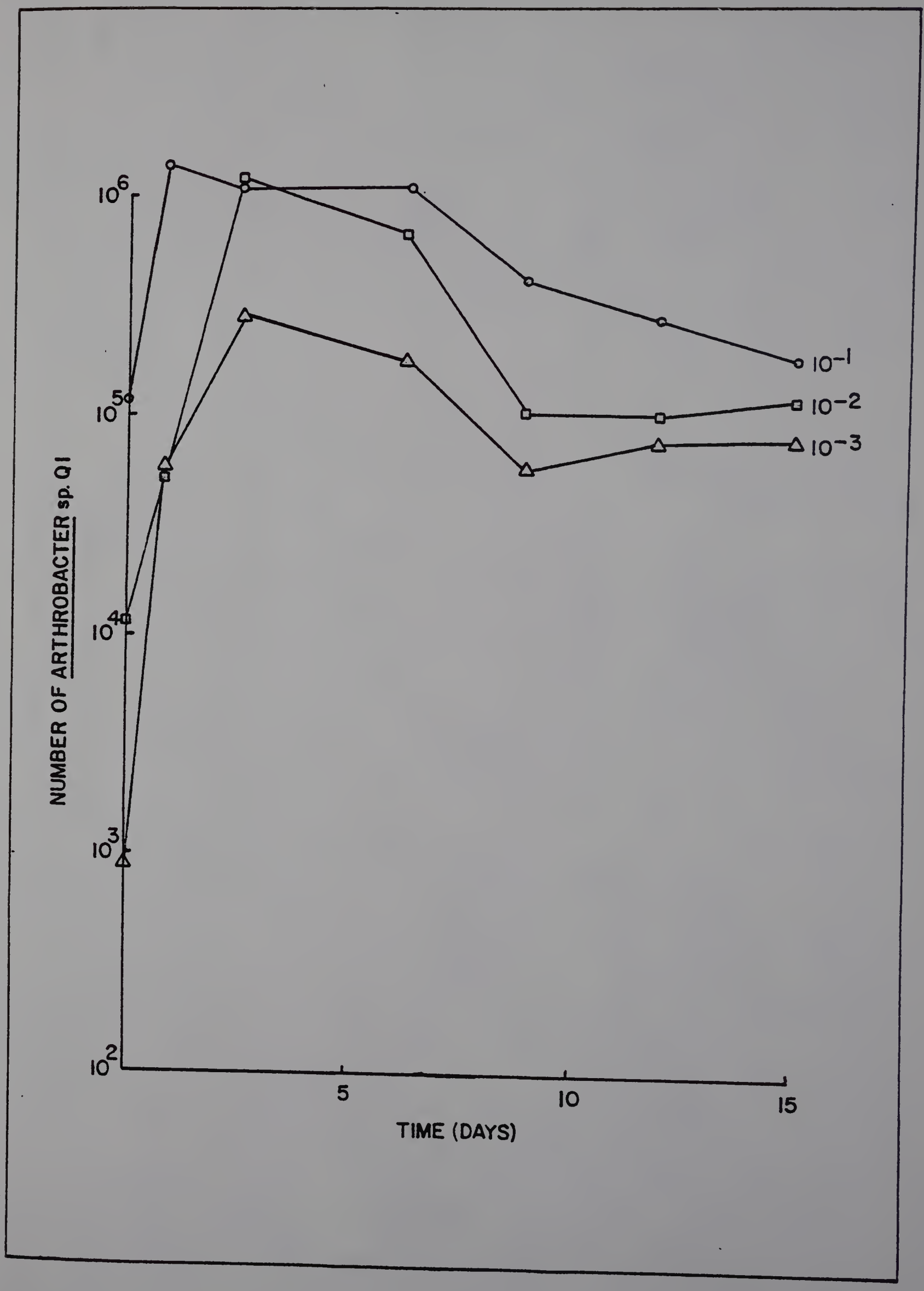
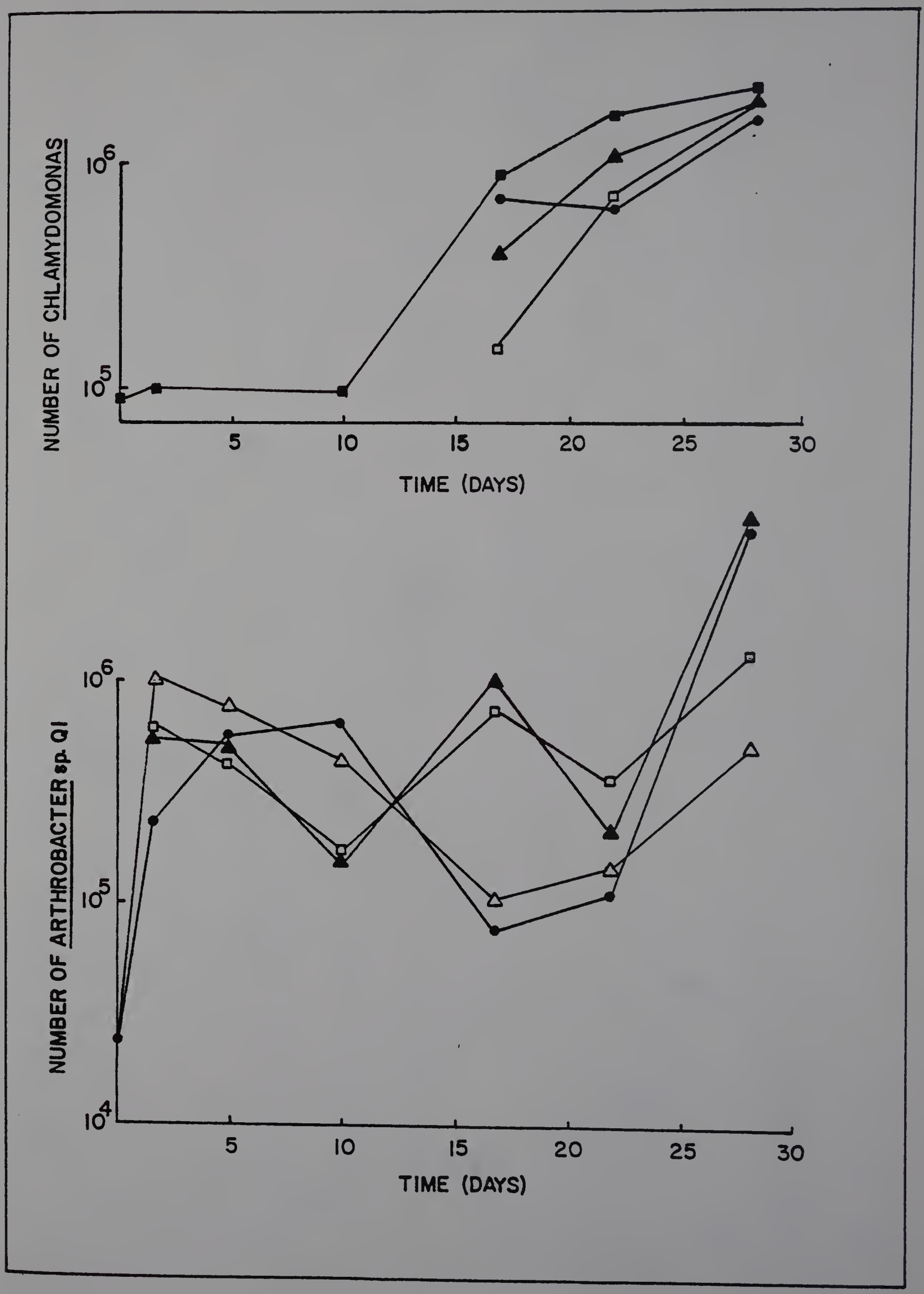

NUMBER OF ARTHROBACTER sp. Q1
10^6
10^5
10^4
10^3
10^2
10^{-1}
10^{-2}
10^{-3}
5
10
15
TIME (DAYS)

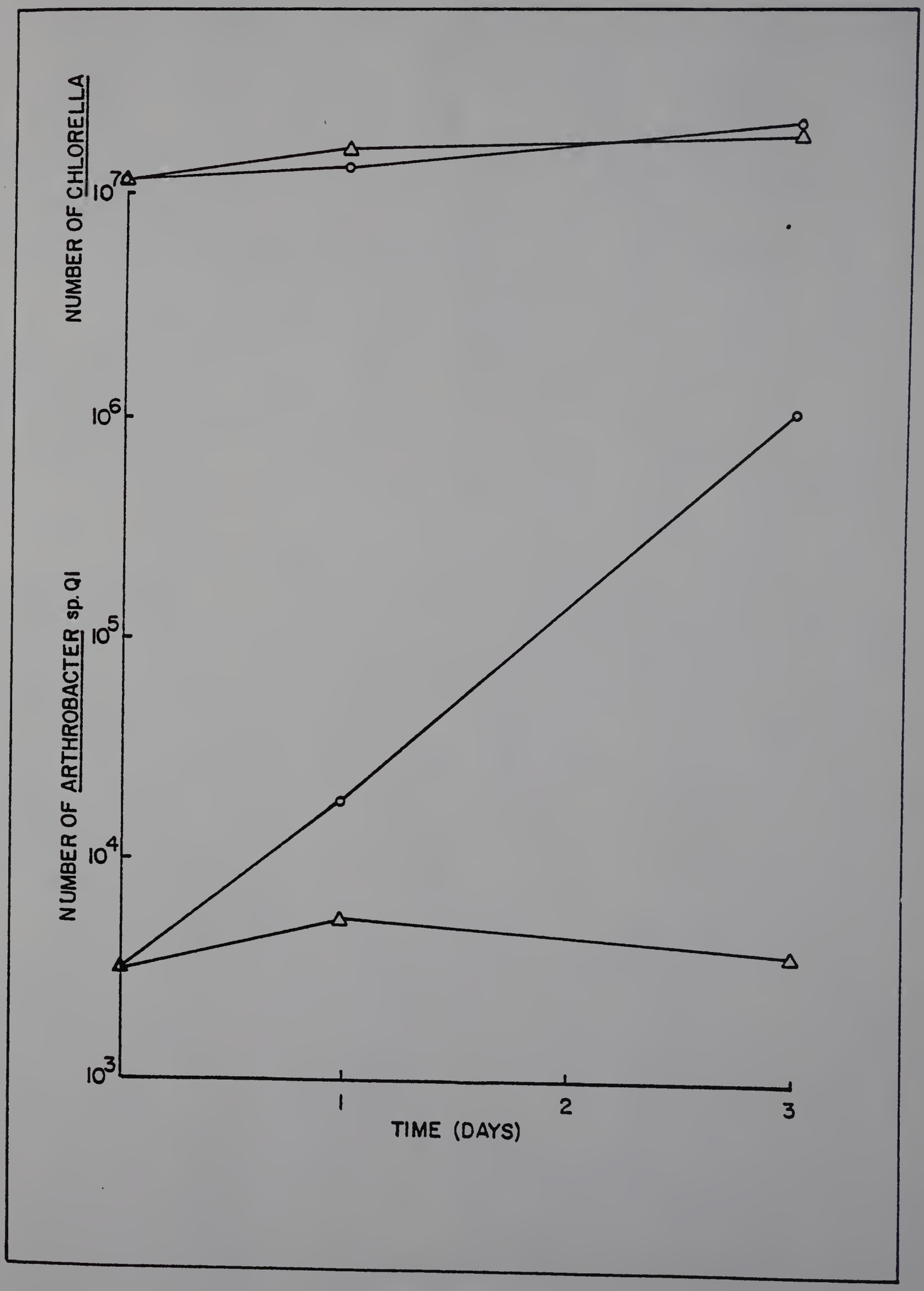
Figure 10. Effects on bacterial and algal growth of equal populations of *Arthrobacter* sp. Q1 added to varying populations of *Chlamydomonas* cells in Bold's Basal Medium. A twice-washed suspension of *Chlamydomonas* was prepared. The 10^{-1} curve represents the growth of a 1/10 dilution of this suspension added to an *Arthrobacter* population. The *Arthrobacter* control and *Chlamydomonas* control are pure cultures. Because algal counts by hemacytometer are not accurate below a population of 10,000 cells per ml, not all algal curves begin at day 0. • represents 10^{-1} dilution; ▲ represents 10^{-2} dilution; ◻ represents 10^{-3} dilution; Δ represents bacterial control; ■ represents algal control.


NUMBER OF CHLAMYDOMONAS
10^6
10^5
5
10
15
20
25
30
TIME (DAYS)
NUMBER OF ARTHROBACTER sp. QI
10^6
10^5
10^4
5
10
15
20
25
30
TIME (DAYS)

Chlorella, and indeed the population of the latter after two weeks (Figure 8) was almost an order of magnitude above that of *Chlamydomonas* after four weeks.

The results of the previous experiments suggest that live *Chlorella* cells are responsible for inhibiting *Arthrobacter* sp. Q1 growth. One possible factor for the bacterial decline was high pH. It had been noted during previous experiments that actively growing *Chlorella* cultures in BBM resulted in pH values between 9.5 and 10. Another possibility for the decrease in bacterial populations was competitive inhibition. To determine whether either of these two factors were involved, a three-week old *Chlorella*-*Arthrobacter* culture (pH 9.9) was apportioned into three parts. When a fresh concentrated volume of BBM was added to two of these samples (this not only provided inorganic nutrients but also reduced the pH to 7.3), an immediate and dramatic rise in the bacterial population was evident (Figure 11). During the three-day experiment, *Chlorella* counts increased only slowly. After this time, the pH of the samples were 8.0 and 8.1. The third sample received no BBM, but sufficient sterile HCl was added to reduce the pH to 7.3. Only a slight rise in bacterial numbers were noted in this case after one day, and thereafter numbers declined. For this sample, the pH was 7.9 after one day and 9.8 after three days. These data indicate that both high pH and competitive inhibition could play a role in the decline of bacterial numbers.

Figure 11. Effects on Arthrobacter sp. Q1 and Chlorella growth of testing an old mixed culture containing these organisms with either a concentrated solution of Bold's Basal Medium sufficient to raise mineral levels to those of the original culture medium, or sufficient HCl to lower the pH to 7.3. Average of two. ° represents BBM addition, Δ represents HCl addition.


NUMBER OF CHLORELLA
NUMBER OF ARTHROBACTER sp. QI
10^7
10^6
10^5
10^4
10^3
1
2
3
TIME (DAYS)

To investigate which of these two factors was more important, the growth pattern of Arthrobacter sp. Q1 was observed in four samples of BBM, each buffered at a different pH. As can be observed in Figure 12, the bacterium grows equally well at pH 6.6, 7.6, and 8.6. Only as the pH approaches 9.6 does bacterial inhibition occur. The results of this and the previous experiment seem to suggest tnat the high pH is less important than the alga's ability to favorably compete with the bacterium for inorganic nutrients.

Chlorella Inhibition by Arthrobacter sp. Q1

When Chlorella-inoculated agar plates (Plate Count Agar) were streaked once with Arthrobacter sp. Q1, a strong wide zone of inhibition occurred around the bacterial streak. This zone failed to appear when the Flavobacterium sp. or Nocardia sp. used in earlier experiments were streaked onto Chlorella-inoculated plates (see Figure 13). Similarly, it did not appear when Chlorella was streaked onto pour plates containing Arthrobacter sp. Q1. The effect was noted whether the plates were incubated in the light or dark. Moreover, the zone of inhibition persisted with time. In peripheral zones of the plates, Chlorella grew well. Undoubtedly the alga was using the glucose in the medium as a carbon source for heterotrophic growth; Chlorella failed to grow in Nutrient Agar, which lacked a carbohydrate.

When strips of agar from the zone of inhibition were

Figure 12. Effect on the growth of Arthrobacter sp. Q1 of Bold's Basal Medium buffered at different pH values.

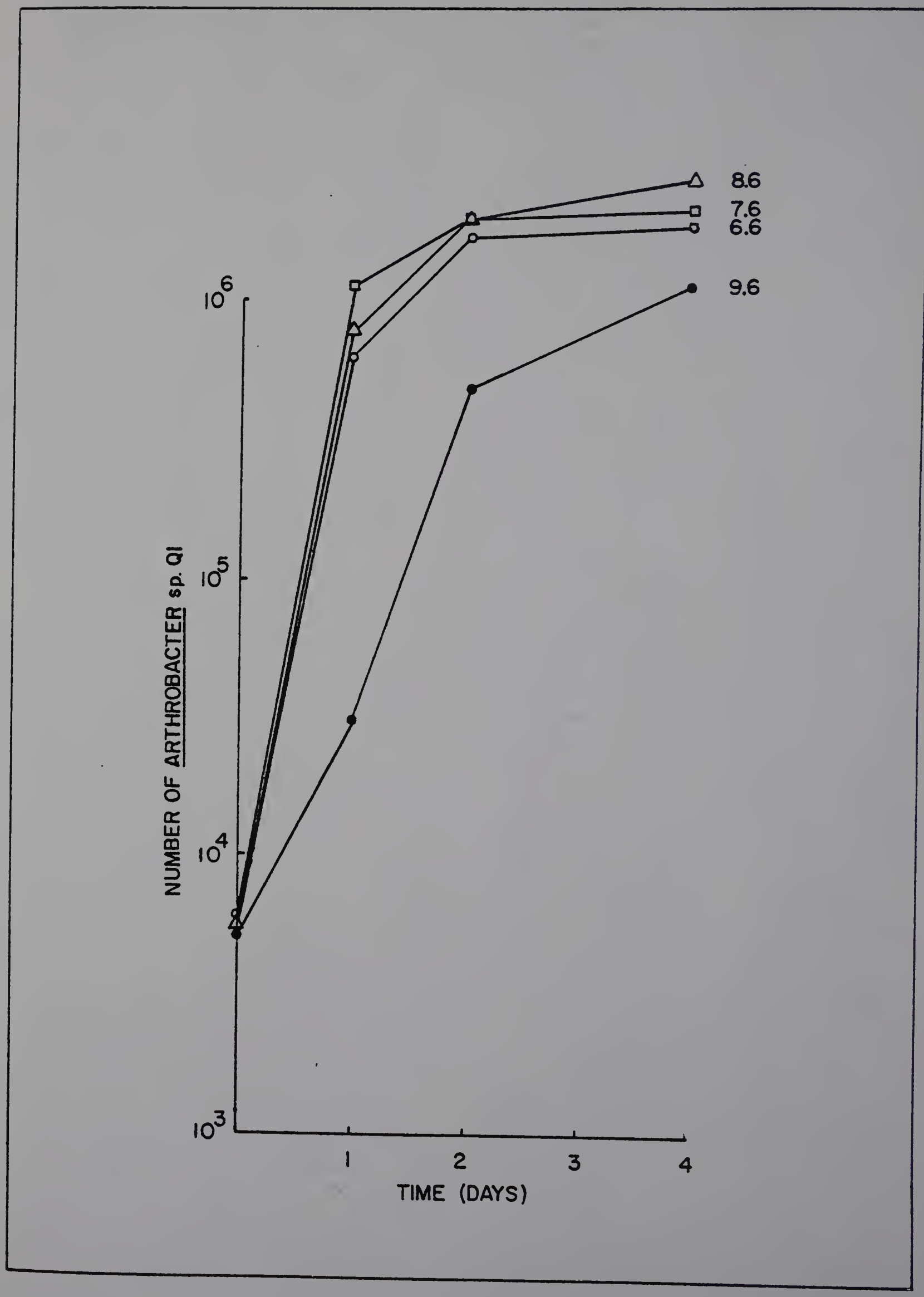

8.6
7.6
6.6
9.6
10^6
10^5
10^4
10^3
NUMBER OF ARTHROBACTER sp. QI
1
2
3
4
TIME (DAYS)

Figure 13. Pour plates of Chlorella in Plate Count Agar streaked once with a bacterial isolate. Zone of inhibition is evident around Arthrobacter streak (center plate) but not around Flavobacterium (upper plate) or Nocardia (lower plate).

transferred to fresh Chlorella-inoculated agar plates and incubated, no evidence of inhibition to the Chlorella was apparent in these plates. Indeed colonies of the alga within the strips appeared. Thus either the Arthrobacter released some substance inimical to algal growth or competitive inhibition produced the effect. Moreover, the growth-restraining factor does not kill the alga but merely inhibits its growth.

To investigate the mechanism of inhibition more closely, Arthrobacter sp. Q1 was subjected to various treatments and then placed in penicylinders on freshly prepared pour plates containing Chlorella. The results are indicated in Table 2. Cell filtrates or dead bacteria failed to inhibit the alga. Only actively growing cells resulted in a zone of inhibition around the penicylinder. In another experiment, when fresh agar strips from the zone of inhibition were crushed with a tissue grinder and added to a penicylinder, no algal inhibition was evident.

One explanation consistent with the previously-mentioned data is that inhibition was caused by bacterial enzymes. To test this possibility, Arthrobacter sp. Q1 was added to three different fresh liquid media containing a large quantity of Chlorella for enzyme induction. The media included Nutrient Broth, BBM containing 0.1% tryptone and 0.01% yeast extract, and unsupplemented BBM. The residue from ultrafiltration from the three cultures (particles above

Table 2. Effects of various treatments of Arthrobacter sp. Q1 cells on Chlorella. Treated material transferred to penicylinders on alga-inoculated agar plates.

TREATMENT	INHIBITION OF ALGAL GROWTH
Actively growing Q1 cells	+
Filtrate from growing Q1 cells[1]	-
Filtrate from disrupted Q1 cells[1,2]	-
Chloroform-treated cells[3]	-
Boiled cells (5 min)[3]	-

[1]Filter sterilized.

[2]Cells disrupted with a French Press.

[3]Cells dead.

a molecular weight of 1000), when filter-sterilized (0.20 micron, Nuclepore) and placed in either penicylinders or holes bored in the Chlorella-inoculated agar plates, failed to produce visually evident inhibition. This eliminated the possibility that stable enzymes or any other stable high molecular weight substance is directly responsible for Chlorella inhibition.

Other reasonable explanations for algal inhibition remain. These include competitive inhibition; unstable enzymes or other unstable high molecular weight substances released by the bacterium; some unstable low molecular weight toxin; and a change in the agar environment (e.g., a pH shift) created by some product released by the Arthrobacter.

Several experiments were performed to determine whether competitive inhibition was a factor in algal inhibition. In one case, Arthrobacter sp. Q1 was streaked once onto Chlorella-inoculated agar plates, and penicylinders were placed within the potential boundary of the zone of inhibition. The addition of the following substances failed to permit algal growth around the penicylinders within this zone: trypticase-yeast extract, glucose, BBM, trace elements from BBM, vitamin B_{12}, $FeSO_4$, K_2HPO_4, KH_2PO_4, and $NaNO_3$. In another case, 2X Plate Count Agar had no effect on the size of the zone of inhibition when compared to normal strength Plate Count Agar. A third experiment involved the transfer of Chlorella-inoculated agar strips from zones of inhibition

onto Noble Agar plates. No other nutrients were thus available to the alga other than the purified agar itself. It was observed that Chlorella grew in these strips. To insure that the Noble Agar itself was not providing nutrients to the alga in the strips, Chlorella was streaked onto other Noble Agar plates. No growth resulted. The results of these experiments indicate that competitive inhibition was not a factor in the algal inhibition observed.

An investigation was then conducted to discover whether a change in pH in the agar caused by bacterial growth could inhibit the Chlorella. No inhibition was found at agar pH values between 5.0 and 8.0; pH values below this range (3.0 and 4.0) and above this range (8.5 and 9.2) were inhibitory only weakly and temporarily. In every case, the algae eventually grew. In a related study, pH was measured over time in m-Plate Count Broth inoculated with Arthrobacter sp. Q1 or two other bacterial isolates unable to inhibit Chlorella. Although the pH in every culture rose slightly, it never rose above 7.4 after six days. Thus pH too was apparently not the causative agent of the algal inhibition.

Attention was next given to determining whether a low molecular weight toxin was responsible for inhibiting the Chlorella. Earlier experiments by J. Rho (personal communication) had revealed that Arthrobacter sp. Q1 and several other lake isolates were heterotrophic nitrifiers. To ascertain whether any correlation existed between nitrification

and algal inhibition on agar plates, both nitrifiers and non-nitrifiers were streaked once onto two types of Chlorella-inoculated agar plates. The results are indicated in Figure 14 and Table 3. Ability to nitrify in a particular medium is indicated by the production of hydroxylamine and nitrite. It can readily be observed that for both Plate Count Agar and Nutrient Agar (with 0.5% glucose added to permit algal growth) a direct correlation exists between algal inhibition and nitrification. Only in the case of P. aeruginosa was there a disparity, but this organism is known to produce other toxins. Interestingly, Arthrobacter sp. Q1 failed to produce a zone of inhibition in Nutrient Agar. That no hydroxylamine or nitrite could be detected in this situation is further evidence of a link between nitrification and Chlorella inhibition.

Besides hydroxylamine and nitrite, other nitrogenous intermediates may also be produced and released by Arthrobacter sp. Q1. An examination was made of a variety of classes of possible intermediates to determine whether any were toxic to Chlorella. All tests were conducted by using Plate Count Agar plates containing Chlorella and 10 µg/ml sample-N. The results are indicated in Figure 15. As can be observed, ammonium, nitrite, nitrate, and the nitro compound proved nontoxic at this concentration. Only free hydroxylamine and the oxime (a type of bound hydroxylamine) proved toxic, with no algal growth at all noted after five

Figure 14. Pour plates of Chlorella in Plate Count Agar streaked once with several bacterial isolates. Isolates inhibiting algal growth are shown by clear zones around the streaks. Organisms are identified clockwise from top. First photograph: Staphylococcus aureus, Bacillus thuringiensis, Nocardia sp., Arthrobacter sp. Q1. Second photograph: Strain P-3, Erwinia carotovora, Strain P-1, Streptomyces sp.

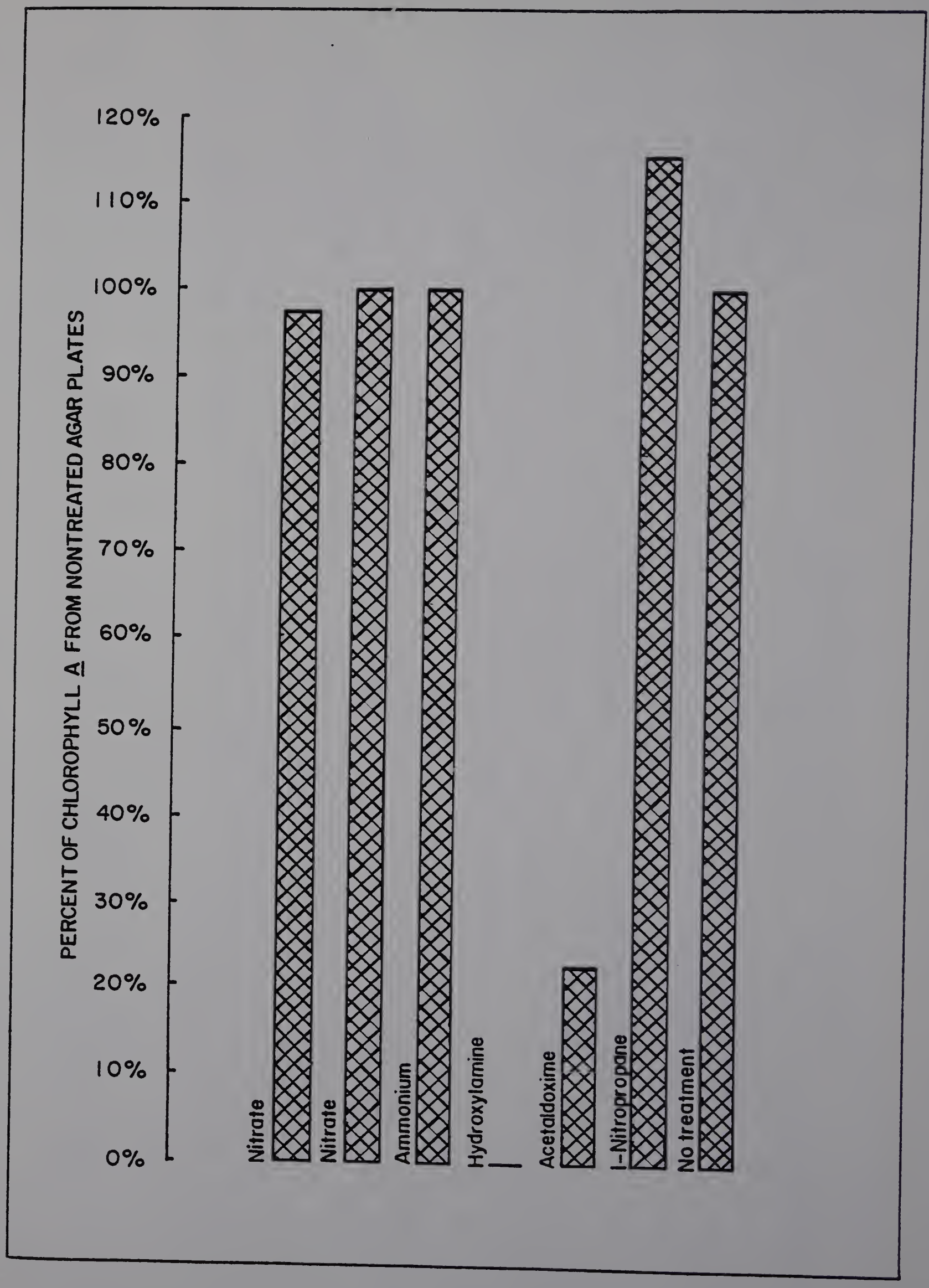
Table 3. Inhibition of Chlorella by intermediates of heterotrophic nitrification.

ORGANISM[1]	PLATE COUNT AGAR			NUTRIENT AGAR[2]		
	ZONE OF INHIBITION	NH_2OH-N	NO_2^--N	ZONE OF INHIBITION	NH_2OH	NO_2^--N
Nocardia sp.	-	-	-	-	-	-
Flavobacterium sp.	-	-	-	-	-	-
Q1	+	+	+	-	-	-
Q2	-	-	-	-	-	-
Q3	-	-	-	-	-	-
P1	+	+	+	+	+	+
P2	+	+	+	+	+	+
P3	+	+	+	+	+	+
P4	-	-	-	-	-	-

[1] "Q" strains isolated from Quabbin Reservoir, "P" strains isolated from Pontoosuc Lake.

[2] 0.5% glucose added to permit Chlorella growth.

Figure 15. Effect on growth of Chlorella in PCA pour plates of various intermediates of nitrification as measured by chlorophyll a concentrations. Initial concentration of all intermediates in the plates was 10 µg/ml sample-N.


PERCENT OF CHLOROPHYLL A FROM NONTREATED AGAR PLATES
120%
110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Nitrate
Nitrate
Ammonium
Hydroxylamine
Acetaldoxime
1-Nitropropane
No treatment

days incubation with the former. This provided initial evidence that Arthrobacter sp. Q1 in oxidizing the reduced organic nitrogen in the medium to hydroxylamine was thereby generating the compound responsible for Chlorella inhibition.

Further studies were performed to provide greater detail on the hydroxylamine-algal interaction. Figure 16 indicates the hydroxylamine concentration over time both within the zone of inhibition and in locations of algal growth. The hydroxylamine-N level within the zone increases to 5 µg/ml four days after bacterial inoculation and then drops. Little if any hydroxylamine was detected outside the zone. In a related experiment, it was found that Arthrobacter sp. Q1 also produced hydroxylamine in agar plates lacking algae. Thus Chlorella is not needed for induction. Figure 17 depicts nitrite-N concentrations in the two locations. Much higher measurements were found within the zone of inhibition than outside the zone. Presumably, the nitrite originates from the oxidation of hydroxylamine.

Because filtrates of Arthrobacter sp. Q1, when placed in penicylinders on Chlorella-inoculated agar, failed to inhibit the alga, it was reasoned that the toxic factor should be expected to decay in the agar with time. That it indeed does is indicated in Figure 18. In this experiment, 10.2 µg/ml hydroxylamine-N was added to both plates containing Chlorella and those lacking the alga, and concentrations of the compound were measured with time. The hydroxylamine

Figure 16. Hydroxylamine-N levels in the inhibited zone and in the non-inhibited zone of Chlorella-inoculated PCA plates streaked once with Arthrobacter sp. Q1. Average of two sets of three plates.

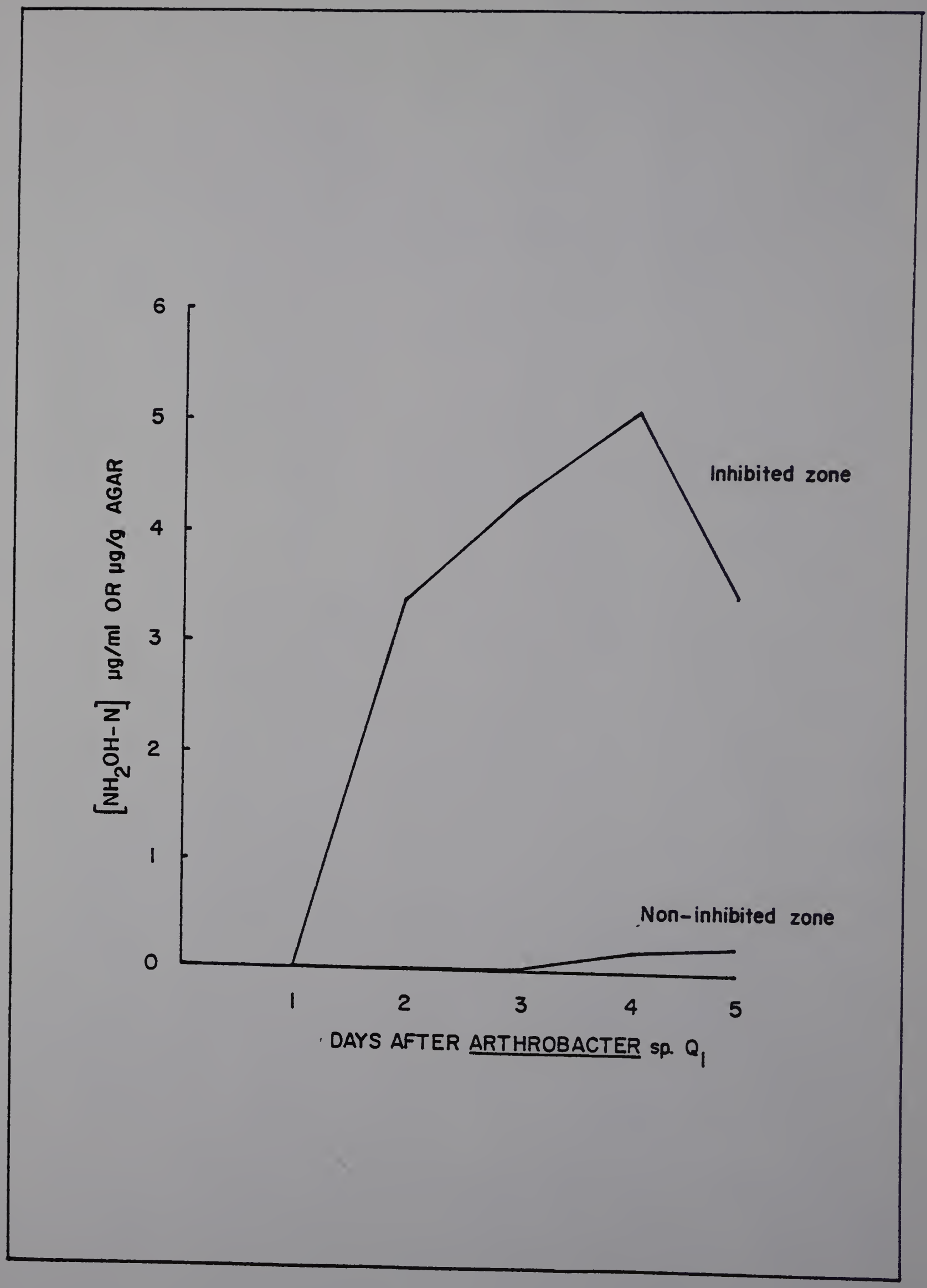

6
5
4
3
2
1
0
[NH2OH-N] µg/ml OR µg/g AGAR
Inhibited zone
Non-inhibited zone
1
2
3
4
5
DAYS AFTER ARTHROBACTER sp. Q1

Figure 17. Nitrite-N levels in the inhibited zone and in the non-inhibited zone of Chlorella-inoculated PCA plates streaked once with Arthrobacter sp. Q1. Average of two sets of three plates.

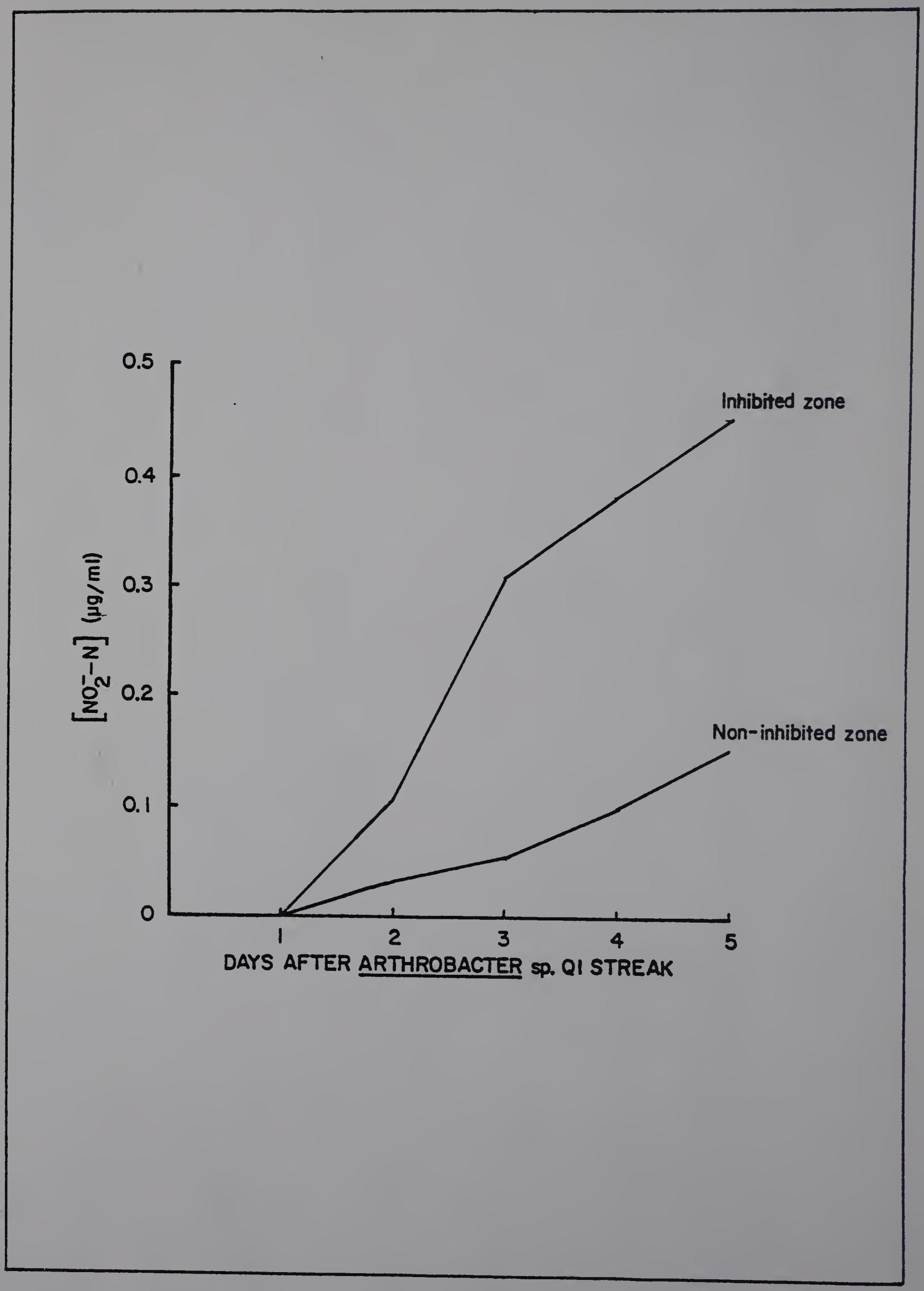
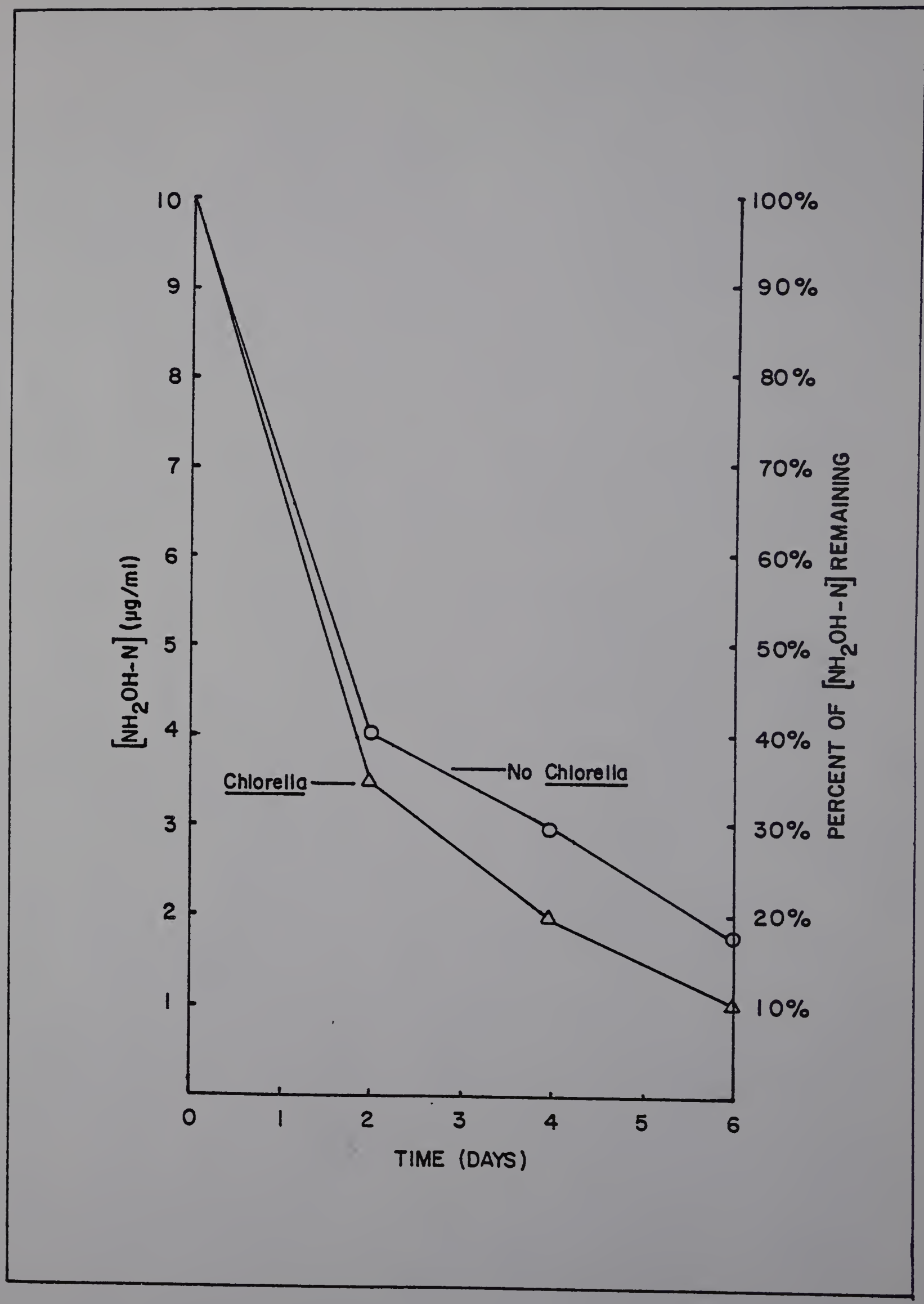

0.5
0.4
0.3
0.2
0.1
0
[NO2−N] (µg/ml)
Inhibited zone
Non-inhibited zone
1
2
3
4
5
DAYS AFTER ARTHROBACTER sp. Q1 STREAK

Figure 18. Decay profile of hydroxylamine in both Chlorella-inoculated PCA plates and uninoculated PCA plates. Average of three plates.


10
9
8
7
6
5
4
3
2
1
[NH2OH-N] (µg/ml)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
PERCENT OF [NH2OH-N] REMAINING
Chlorella
No Chlorella
0
1
2
3
4
5
6
TIME (DAYS)

levels dropped to about one-half the initial concentration after about 1½ days in both cases. The subsequent drop was somewhat greater in Chlorella-inoculated plates, and eventually (about seven days) Chlorella colonies were observable on the plates without the aid of a microscope.

The possibility that water evaporation from the agar was triggering the free hydroxylamine decrease by increasing the ratio of attachment sites on organic carbon skeletons over attachment sites on water molecules was examined. Figure 19 shows that during the first 1½ days, the moisture lost from agar was only about 5%. Thus this effect probably cannot account for the hydroxylamine decay. While substantial oxime formation may still occur, the oxidation or chemical dissociation of hydroxylamine seems a more reasonable explanation for its decay.

In previous experiments it had been noted that 10 µg/ml hydroxylamine-N inhibits algal growth (Figure 15) and that Arthrobacter sp. Q1 produces about 5 µg/ml hydroxylamine-N. The next series of experiments were performed to determine whether indeed this lower value was adequate to block algal growth, and, more generally, whether a threshold hydroxylamine concentration existed inimical to this growth. Figure 20 depicts chlorophyll a concentrations in agar plates containing increasing initial concentrations of hydroxylamine. Incubation time was seven days. It can be seen that the levels of hydroxylamine-N resulting from bac-

Figure 19. Water loss in uninoculated PCA plates. Average of four plates.

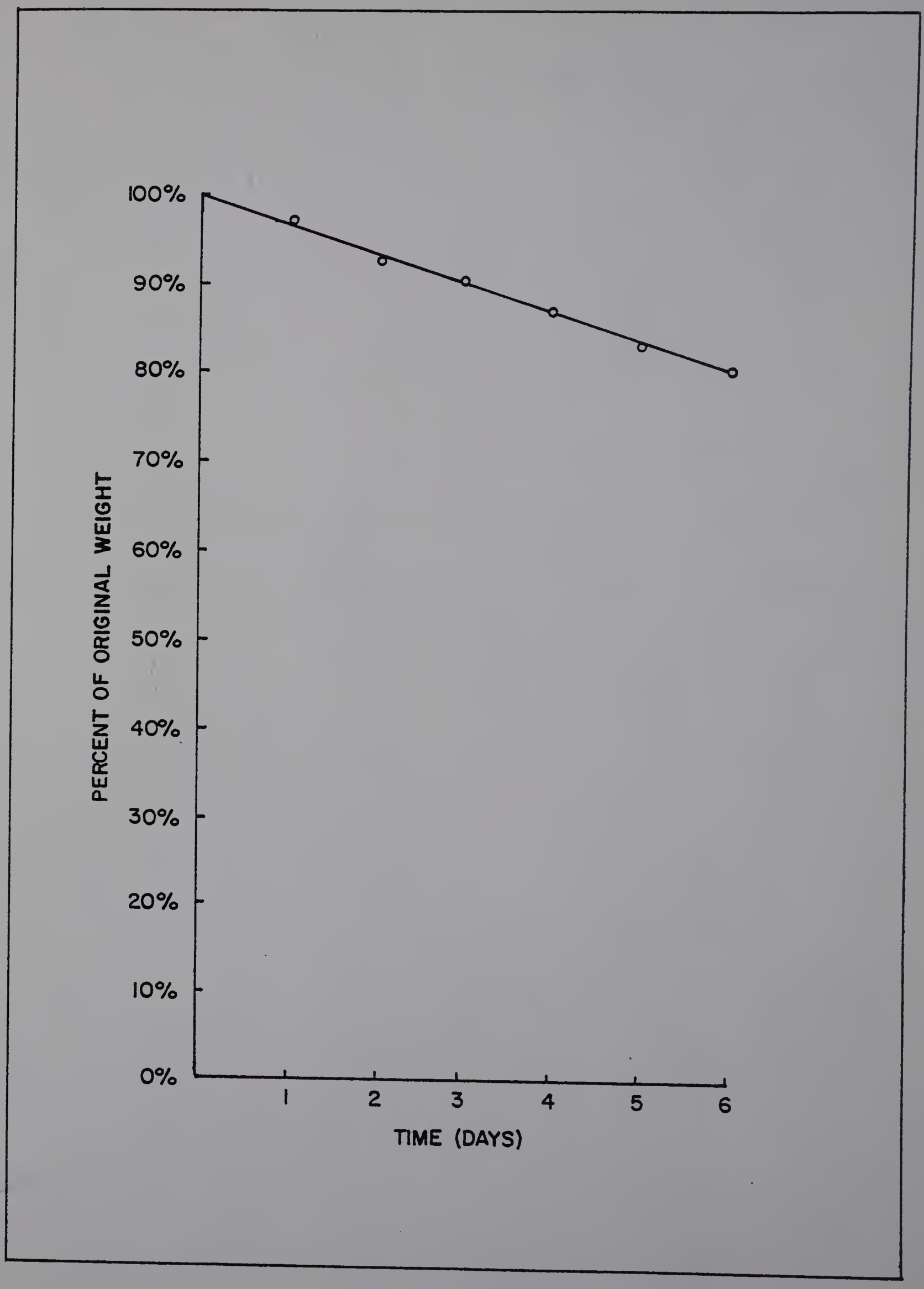
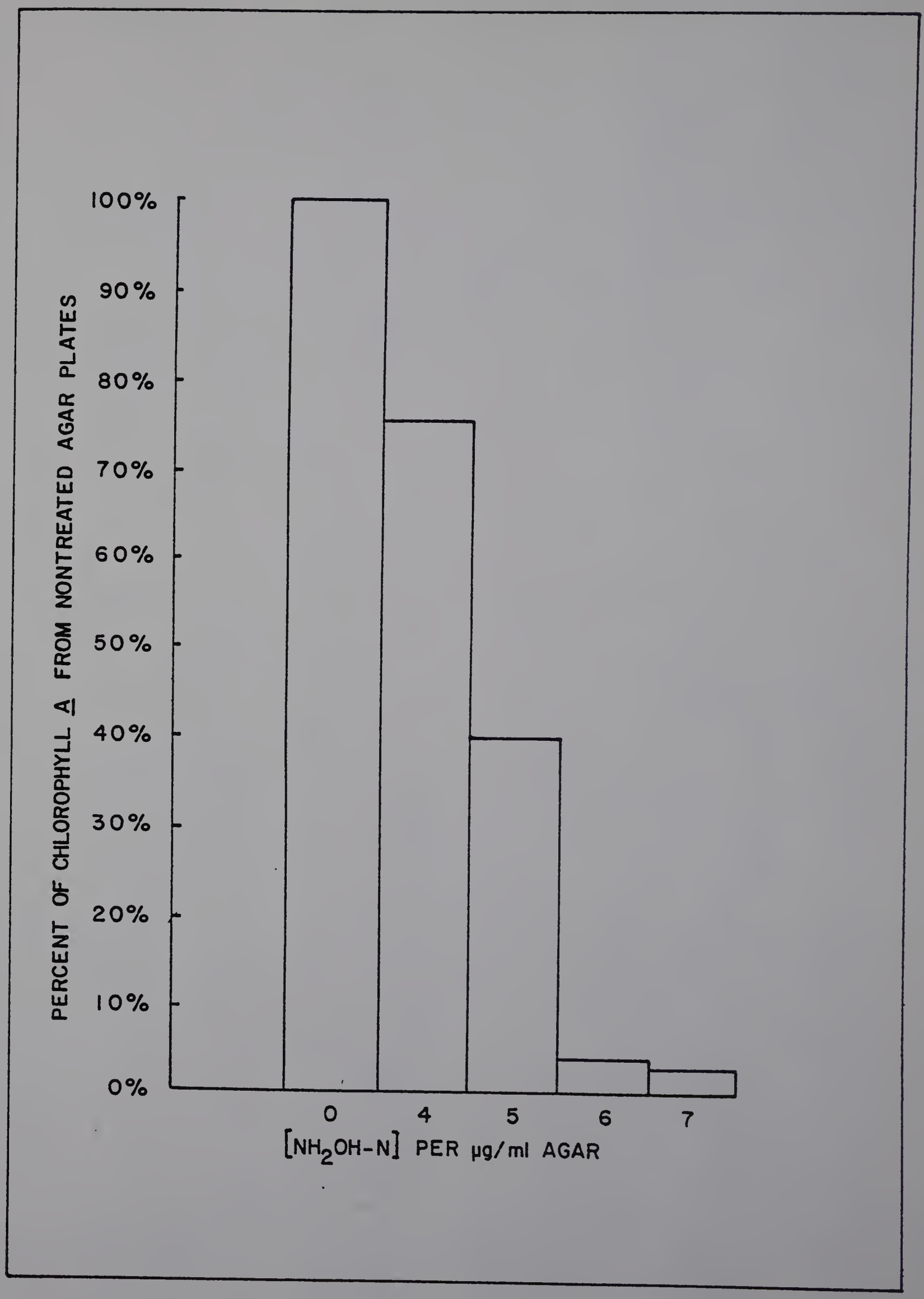

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
PERCENT OF ORIGINAL WEIGHT
1
2
3
4
5
6
TIME (DAYS)

Figure 20. Comparison of chlorophyll a concentrations found in Chlorella-inoculated plates treated with different concentrations of hydroxylamine.


PERCENT OF CHLOROPHYLL A FROM NONTREATED AGAR PLATES
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
4
5
6
7
[NH2OH-N] PER µg/ml AGAR

terial growth is sufficient to inhibit Chlorella growth. Using the data in Figure 18, the seven-day incubation time should have reduced hydroxylamine levels to less than 10% of the initial concentration. Since green Chlorella colonies did eventually appear on all plates, some tolerance to low concentrations is evident.

In contrast to the results with hydroxylamine, increasing levels of nitrite seemed to slightly enhance algal growth (Figure 21). The range of concentrations tested were selected on the basis of nitrite levels found in the zone of inhibition. The enhancement in growth again confirms that nitrite is not the causative agent of algal inhibition.

Further experiments were conducted to determine the ability of hydroxylamine to inhibit algal growth in an environment more similar to fresh water than agar medium. In tubes of BBM (see Figure 22) inhibition is evident at an initial hydroxylamine-N concentration of 0.16 µg/ml. Figure 23 represents the decay profile of hydroxylamine in BBM. It is apparent that one-half the initial level is lost after about 1½ days, similar to that in agar. Therefore it is probable that Chlorella cells are inhibited by considerably lower concentrations of hydroxylamine-N than 0.16 µg/ml.

A critical consideration was the specificity of the Magee-Burris test for hydroxylamine. The inhibition factor had been identified as hydroxylamine on the basis of this test. The importance of cross reactions was determined using

Figure 21. Comparison of chlorophyll a concentrations found in Chlorella-inoculated plates treated with different concentrations of nitrite.

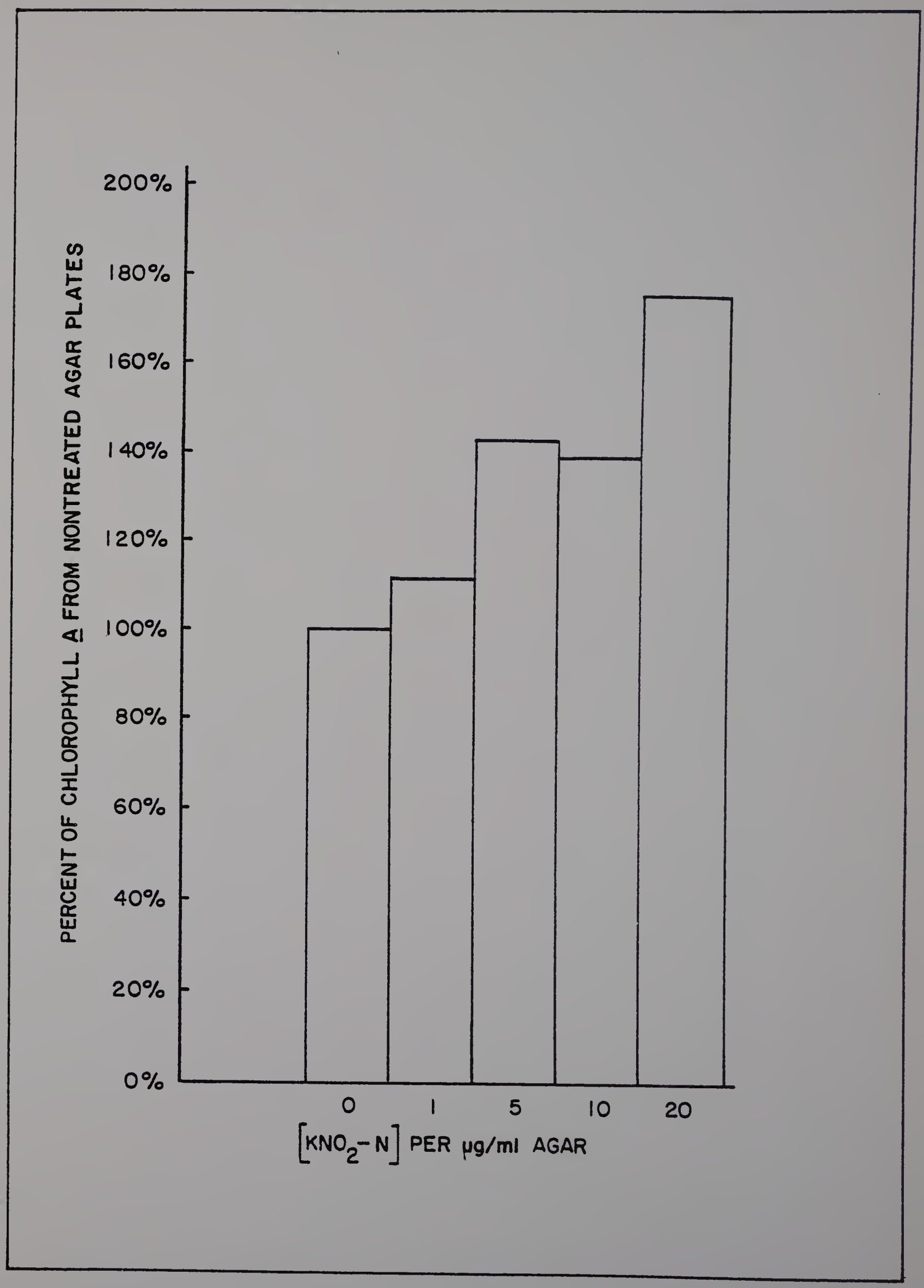

PERCENT OF CHLOROPHYLL A FROM NONTREATED AGAR PLATES
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
1
5
10
20
[KNO2-N] PER µg/ml AGAR

Figure 22. Growth of axenic Chlorella culture in Bold's Basal Medium treated with different concentrations of hydroxylamine. Incubation time was seven days under fluorescent lighting. Average of two tubes.

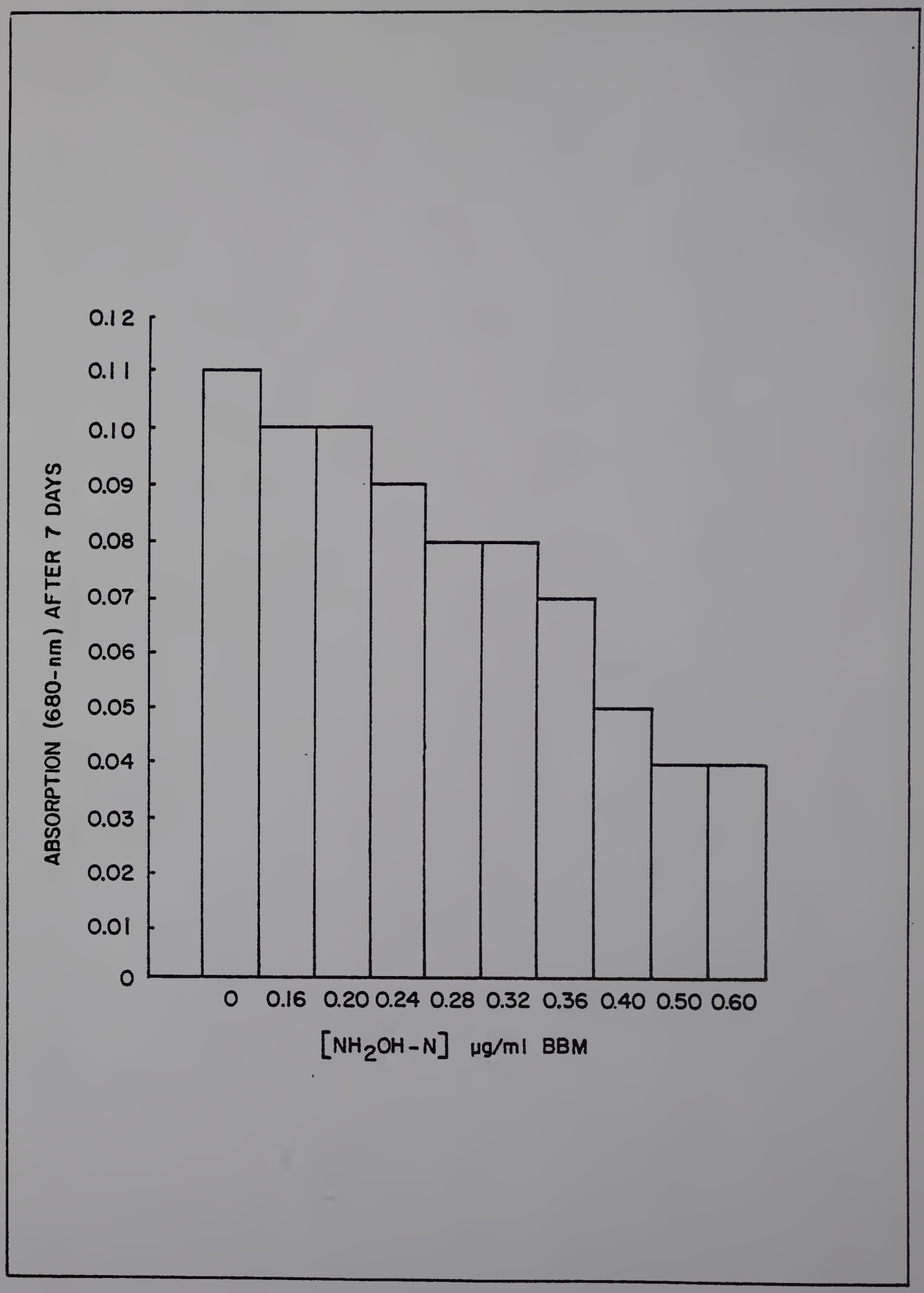
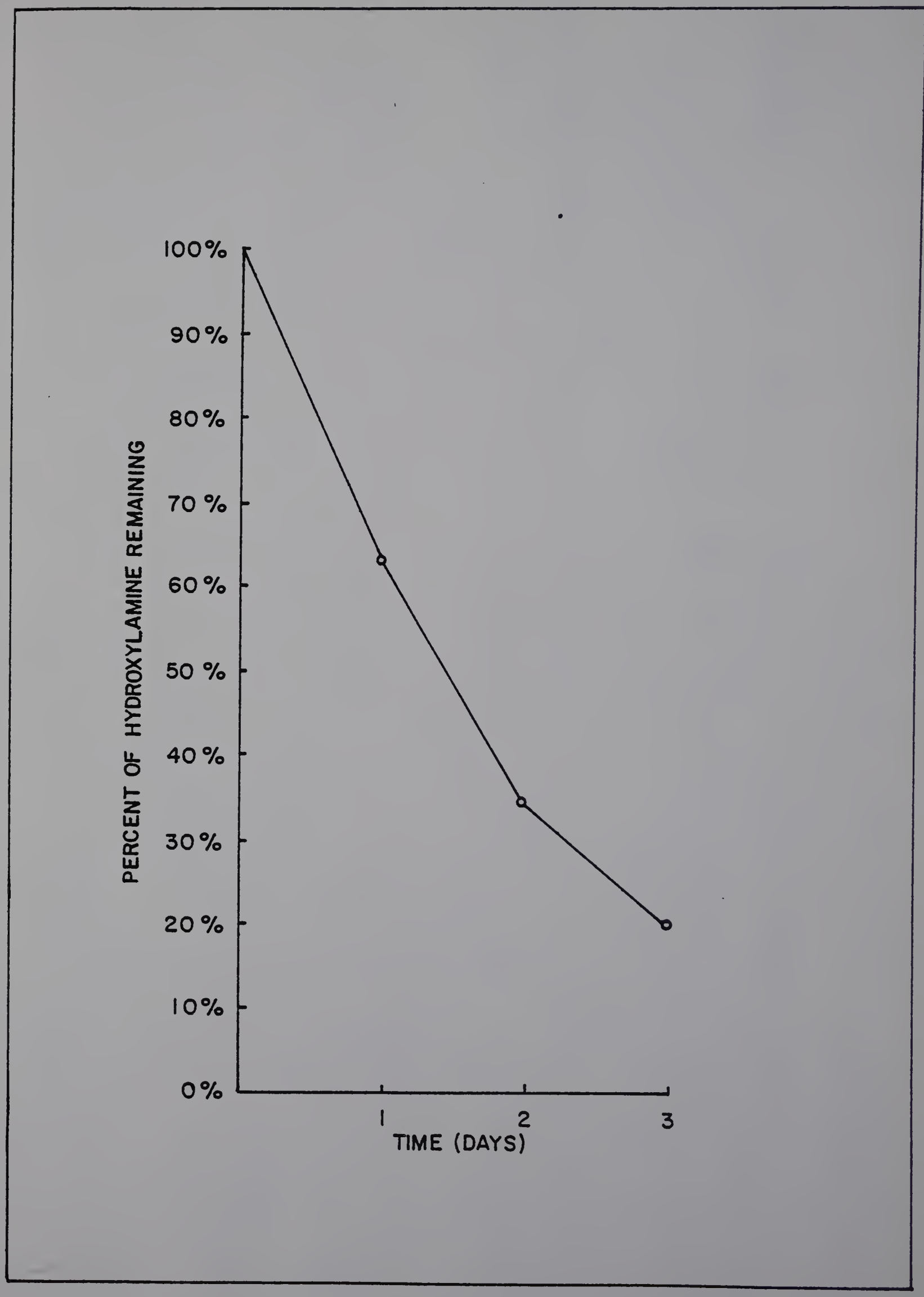

ABSORPTION (680-nm) AFTER 7 DAYS
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0 0.16 0.20 0.24 0.28 0.32 0.36 0.40 0.50 0.60
[NH2OH-N] µg/ml BBM

Figure 23. Decay profile of hydroxylamine in sterile Bold's Basal Medium.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
PERCENT OF HYDROXYLAMINE REMAINING
1
2
3
TIME (DAYS)

various possible intermediates in nitrification. The results are indicated in Table 5 (Appendix A). Although high concentrations were used, none of the chemicals tested except acetaldoxime (and hydroxylamine) produced a positive reading for hydroxylamine in any of the three media used.

Because of the false positive indication of acetaldoxime, an expanded and more quantitative study on oxime was deemed necessary. Table 6 (Appendix A) represents the results of this experiment. According to the data, slightly over one-third of the hydroxylamine added was detected in the broth and in agar plates as hydroxylamine. This is more than one order of magnitude greater than that indicated by the oximes. Thus while oximes do produce a positive hydroxylamine test, the interference is relatively unimportant. Indeed because of an equilibrium between the free and bound hydroxylamine, the positive tests for the latter may actually represent free hydroxylamine levels.

Several other methods were attempted to strengthen the conclusion that hydroxylamine was the alga-inhibiting factor on agar plates. In one such attempt, various known chemical inhibitors of autotrophic nitrification were added to ammonium-acetate medium to determine if nitrification by Arthrobacter sp. Q1 could be blocked. As Table 7 (Appendix B) indicates, none of the chemicals used in concentrations allowing Arthrobacter growth blocked hydroxylamine production. In another test, Fe (as $FeCl_3$) was added in the

preparation of Chlorella-inoculated agar plates such that its final concentration was 2 µg/ml. The magnitude of the zones of inhibition around the Arthrobacter streak did not depart from that previously observed. Thus the possible class of nitrification intermediates known as hydroxamic acids, which is produced in an iron-deficient medium, is unlikely as the causative agent of the zone of inhibition.

In a third test, it was reasoned that higher glucose levels in the agar plates would result in a higher C:N ratio, thus increasing the amount of reduced nitrogen needed for assimilation. The lower levels of reduced nitrogen available for oxidation would conceivably result in smaller zones of inhibition. When Chlorella-inoculated agar plates were supplemented with various concentrations of glucose, this expectation was borne out. The zone of inhibition around the Arthrobacter was greatest at the 1% glucose level than at higher levels (see Table 4). An analysis of variance indicated that the perimeters of the zones of inhibition were significantly different (99.5% confidence level) in the cases 1% as opposed to the 3% and 5% as opposed to the 10% glucose levels. The difference in perimeters between 3% and 5% levels was not significant.

Another method for bolstering the theory of hydroxylamine-mediated algal inhibition in the agar was based on the observation that Arthrobacter sp. Q1, when mixed with a culture of another lake isolate and then streaked once onto

Table 4. Area of zone of inhibition of heterotrophically growing Chlorella in the presence of Arthrobacter sp. Q1 at different glucose concentrations. Average of three plates per glucose level.

% GLUCOSE	AREA OF ZONE (mm^2)
1	197
3	112
5	107
10	79

Chlorella-inoculated agar plates, failed to produce the expected zone of inhibition. Reasoning that the second bacterium was metabolizing the inhibiting factor produced by Arthrobacter, presumably hydroxylamine, then Chlorella-inoculated plates possessing a high concentration of hydroxylamine and streaked once with this bacterial isolate should show algal growth--at least initially--only in the vicinity of this streak. When the experiment was performed using plates containing 12 µg/ml hydroxylamine-N, this effect was easily discernible (see Figure 24).

In an attempt to determine if a Chlorella culture grown in BBM could supply a sufficient amount of organic material to permit the production of detectable levels of nitrification products by the Arthrobacter sp. Q1, the two organisms were inoculated into this medium in which the nitrate was replaced by a comparable concentration of ammonium. Neither hydroxylamine nor nitrite was observed, although the Chlorella grew well in this medium.

Figure 24. Effect of a hydroxylamine-metabolizing bacterium on Chlorella-inoculated PCA plates containing 12 µg/ml hydroxylamine-N. The algal colonies are observed growing only around the bacterial streak.

DISCUSSION

Effects of Algae on Bacteria

It is apparent from these studies that bacteria and algae interact in a highly selective manner. Bacterial populations which were associated in nature with an algal species generally flourished in *in vitro* studies, while one that was not, namely *Serratia marcescens*, quickly disappeared, presumably because of inferior competitive attributes in comparison with algal-associated flora or by the restrictive action of algal products themselves. The response of *Arthrobacter* sp. Q1 to different axenic live algae varied greatly--from stimulation (*Ankistrodesmus*) to indifference (*Chlorococcum*) to inhibition (*Chlorella*). Filter-sterilized algal filtrates were all strongly stimulatory to the *Arthrobacter* and two other bacteria isolated from lakewater, but even in these instances a difference in response among the organisms was noted. These findings provide further support to the conclusions of Bell and Mitchell (1972) and Bell et al. (1974) that algal-mediated bacterial selection may be occurring in natural waters. In their 1974 study, these investigators found that in a simulated marine environment a species of *Pseudomonas* assimilated the extracellular products of the alga *Skeletonema costatum* at a rate forty times great-

er than a Spirillum species. In their 1972 study, they found marine bacterial isolates were dissimilarly chemotactic to filtrates from algal cultures, but overall the threshold concentration for attraction was significantly higher than that found in natural seawater.

The only alga which clearly inhibited the Arthrobacter in this investigation was Chlorella vulgaris. It failed even to stimulate the bacterial contaminants present in the original culture. It has long been recognized that Chlorella vulgaris can at times produce and liberate a substance (chlorellin) which retards not only its own growth, but also the growth of many gram-positive and gram-negative bacteria (Pratt and Fong, 1940; Pratt et al., 1944). Scutt (1964) concluded that liberation of the inhibitor, apparently a peroxide produced by the photo-oxidation of unsaturated fatty acids, was not a general phenomenon with Chlorella but only occurs under certain conditions. This mechanism of bacterial toxicity evidently was not involved in the present investigation. Arthrobacter sp. Q1 counts, for example, decreased during a period of increasing Chlorella numbers (Figures 7 and 8). This argues strongly against the presence of the autolytic agent. So does the fact that the Arthrobacter was catalase positive, responded immediately to the addition of inorganic salts in an aged Chlorella-bacterial culture, and grew quite well in a Chlorella filtrate. Thus another agent must be responsible for Arthrobacter inhibi-

tion. In another study, Vela and Guerra (1966) demonstrated that Chlorella pyrenoidosa could inhibit the growth of many species of bacteria, but no evidence was presented to indicate the presence of an autolytic agent. In that investigation, those species of bacteria inhibited by the actively growing algae could not grow in algal filtrate. Since the Arthrobacter (as well as the Flavobacterium and Nocardia) in our study grew well in Chlorella filtrate, another inhibition factor must be responsible.

Evidence also exists that some strains of Chlorella can stimulate bacteria, at least under certain conditions. Nalewajko et al. (1976) found that the growth of a mixed culture of bacteria and also Pseudomonas fluorescens were stimulated by the presence of Chlorella pyrenoidosa Chick cultures. Their experiments, however, were terminated after only a few hours unlike our studies which continued for many weeks. Maksimova and Pimenova (1969b) observed that bacteria were six to seven orders of magnitude greater in the presence of each of two strains of Chlorella vulgaris and one strain of Chlorella pyrenoidosa after 3-10 days growth. None of the bacteria used, however, were Arthrobacter. There is ample evidence in the literature that Chlorella vulgaris does liberate extracellular products (Maksimova et al., 1965; Maksimova and Pimenova, 1969a).

In contrast to the great effect Chlorella had on bacteria, Arthrobacter sp. Q1 failed to noticeably affect

Chlorella growth. This observation in inorganic medium is consistent with that of other investigators (Maksimova and Pimenova, 1969b; Maksimova and Lastochkina, 1964).

The results of this study indicate that the filter-sterilized filtrates of Chlorella, Chlamydomonas, Chlorococcum, and Ankistrodesmus all strongly stimulate Arthrobacter sp. Q1. Yet in live cultures, only Ankistrodesmus noticeably stimulated the Arthrobacter, and less so Chlamydomonas. Chlorella strongly inhibited Arthrobacter, the effect becoming more pronounced with time. A toxic substance was apparently not involved in this inhibition. Neither was high pH directly associated with Arthrobacter mortality. This latter observation is consistent with that of Maksimova and Lastochkina (1964) and Verstraete and Alexander (1973). In the latter study, Arthrobacter growth was not affected at pH 9. The high pH in the present investigation, however, may have played an important indirect role. According to Wetzel (1975), the availability of inorganic ions such as iron, phosphate, manganese, and various micronutrients are strongly regulated by redox conditions and pH. At conditions of high pH and redox levels such as occurred in our investigation, these ions are substantially less soluble than the reverse situation. Much of the iron, phosphate, and other ions in the BBM culture not assimilated by the microorganisms could have precipitated. This could explain the prompt and vigorous growth of the Arthrobacter upon the

addition of inorganic nutrients.

While the decreasing availability of inorganic substances could have accounted for the decline of the Arthrobacter population, it cannot explain the continued growth of Chlorella. This anomaly might be explained in several ways. Lange (1976) suggested that algal sheaths retained and concentrated essential nutrients. While his investigation involved blue-green algae which are known to possess large gelatinous sheaths, it may have been operable to a lesser extent with Chlorella.

A better explanation for concurrent Arthrobacter decline and Chlorella growth involves competitive inhibition at high pH. Chlorella may have a greater affinity for one or more of the critical inorganic nutrients than Arthrobacter at higher pH values. This is supported by the dramatic increase in numbers of Arthrobacter and two other bacterial species in the Chlorella filtrate, where algal competition was not an issue. Bagnyuk et al. (1976) reported that pure cultures of Chlorella vulgaris and Scenedesmus quadricauda were able to quickly remove iron ions from solution by both assimilation and by precipitation. Maksimova and Fedenko (1965) found that the growth of Chlorella vulgaris was accompanied by an increase in redox potential, and that this led to a sharp decrease in the numbers of Pseudomonas ovalis and Bacillus cereus isolated from this alga. Artificial lowering of the redox potential completely reversed the toxic

effect of the alga. Both of these reports are consistent with the view that at high redox potentials and pH, Chlorella outcompetes Arthrobacter for inorganic nutrients.

According to Wetzel (1975), the pH of most natural lakes ranges from 6 to 9, but can extend to 12. Hardwater lakes buffered by the bicarbonate system are strongly buffered at pH values above 8. Softwater lakes are generally more acidic and less well buffered. Under the influence of strong photosynthesis, lakes can undergo large diurnal fluctuations in pH, with late afternoon values often exceeding 10. Our data suggest that some algae can successfully compete in this environment for inorganic substances to the detriment of some indigenous bacteria.

The variation in Arthrobacter response to different algal species may be due to dissimilarities among the algae in their tolerance to high pH values. It is noteworthy that Kroes (1971) found that Chlorococcum (which failed to stimulate Arthrobacter sp. Q1 in our investigation) inhibited the growth of Chlamydomonas (which weakly stimulated the Arthrobacter) only in unbuffered medium, and concluded that pH was very important in relations between species. He was uncertain, however, whether the pH effect was direct or whether it acted indirectly (e.g., by means of variation in salt availability).

Chlorella Inhibition by Arthrobacter sp. Q1

In our investigation, Chlorella grew well in the dark on solid organic media containing glucose. Inhibition of the alga under these conditions resulting from the growth of Arthrobacter sp. Q1 was not due to competitive inhibition. Direct contact between the alga and bacterium was not necessary for algal inhibition as it was in other studies (Stewart and Brown, 1969; Shilo, 1970; Gromov et al., 1972). No evidence was obtained that more stable enzymes and high molecular weight compounds were directly involved, even though bacterial filtrates were concentrated three to twenty times the levels in the original sample. It is interesting that some enzymes thought to be involved in nitrification such as catalase and peroxidase (Keilin and Nicholls, 1958) are among those considered quite stable (Worthington Enzyme Manual, 1972). Thus evidence was strong that a low molecular weight compound (below 1000) was involved in Chlorella inhibition. The most likely candidate was demonstrated to be hydroxylamine, produced by the Arthrobacter sp. Q1.

In general, microbiologically-mediated hydroxylamine production is discussed in terms of nitrification, but Jensen (1951) and Tanaka (1953) suggest that denitrification and other processes of nitrite and nitrate reduction might also be involved. Nitrogen fixation can also be a source of hydroxylamine (Jones, 1973). A number of heterotrophs have

been shown to produce hydroxylamine (see Tables 1 and 3). Arthrobacter sp. Q1 was found to accumulate over 30 µg/ml hydroxylamine-N after six days in ammonium-acetate medium (for constituents of this medium, refer to Appendix C). Hydroxylamine production by this organism was not blocked by compounds effective in restricting production in autotrophs (Table 7, Appendix B). This is consistent with the findings of Verstraete and Alexander (1972a), who concluded that the mechanisms of heterotrophic nitrification and autotrophic nitrification differ.

In contrast to its activity in ammonium-acetate medium, the Arthrobacter sp. Q1 failed to produce any detectable hydroxylamine in BBM with Chlorella cultures, and failed to nitrify when the nitrate normally in BBM was replaced with ammonium. Thus organic carbon levels must play a critical role in nitrification. Chlorella alone was unable to supply the levels required for heterotrophic nitrification.

There are several reports in the literature indicating the presence of hydroxylamine in lakes. Tanaka (1953) found this compound near the bottom of a mesotrophic lake under anoxic conditions in the autumn but not in June. Evidence of its presence was also found in a moderately alkaline European crater lake (Baxter et al., 1973). Free hydroxylamine is also present in stagnant water due to the bacteriological reduction of nitrite (Jones, 1973).

Several publications have examined the tolerance of specific microorganisms to hydroxylamine. According to the Merck Index (Windholz, 1976), the latter is moderately toxic, a known mutagen. Castell and Mapplebeck (1956) cite several articles which indicate generally that concentrations between 1 and 250 µg/ml of hydroxylamine are required for inhibition of bacteria, depending on the species. Their own data indicate that a concentration of 20 to 30 µg/ml allowed growth of most of the 119 bacterial cultures used, and several fungi and yeasts tolerated 200 µg/ml hydroxylamine. In our investigation, 12 µg/ml hydroxylamine (5 µg/ml hydroxylamine-N) was detected after four days in agar plates streaked with Arthrobacter sp. Q1. From the previous study most bacteria, yeasts, and fungi should have been able to tolerate this concentration. Yet Chlorella was strongly inhibited. According to Figure 22, Chlorella grown in BBM under continuous lighting is inhibited by 0.38 µg/ml hydroxylamine (0.16 µg/ml hydroxylamine-N). This suggests that hydroxylamine is a selective inhibitor for at least some strains of algae. The fact that Tanaka (1953) found an order of magnitude less hydroxylamine in a lake further suggests that any Chlorella inhibition would be limited to localized areas in lakes high in organic nutrients.

Hydroxylamine is very unstable in aqueous solutions. This was noted in Figures 18 and 23. It decomposes to ammonia and either dinitrogen (alkaline conditions) or nitrous

oxide (acid conditions) (Jones, 1973). The instability of hydroxylamine may account for the lack of inhibition of Chlorella by filtrates from Arthrobacter sp. Q1 (see Table 2). According to Rajendran and Venugopalen (1976), the decomposition is pH-dependent, being much more stable in acid solutions than in alkaline.

The identification of hydroxylamine was based on the 8-hydroxyquinoline method of Magee and Burris (1954), a very sensitive test. It is possible for hydroxylamine to bind with a carbon skeleton (especially ketones and aldehydes) to form what is often referred to in the literature as a bound hydroxylamine. The latter includes oximes (Marshall and Alexander, 1962), hydroxamic acids (Marshall and Alexander, 1962), nitroso compounds (Verstraete and Alexander, 1972a), and other compounds. As noted in Appendix A, oximes may to a degree interfere with the test for hydroxylamine and possibly the other bound hydroxylamines may do so as well. The method commonly used to differentiate free hydroxylamine from bound hydroxylamine is that of Novak and Wilson (1948), which involves heating an acidified sample in a boiling water bath. This treatment liberates the free hydroxylamine, which is then quantified and compared to free hydroxylamine levels measured prior to the treatment. Unfortunately oxime and hydroxylamine standards in m-Plate Count Broth or Plate Count Agar failed consistently to yield a positive hydroxylamine reading after boiling in acid; in

contrast, standards in water samples did produce the expected concentrations when boiled. Thus a bound hydroxylamine could not be definitely excluded from consideration as the Chlorella-inhibiting factor.

Indeed there is some circumstantial evidence that at least part of the hydroxylamine liberated in the agar plates reacted with the constituents in the plate medium to produce an oxime. It is well known (e.g., Siggia, 1963) that hydroxylamine can react with an aldehyde or ketone to form an oxime according to the following formula:

Both Siggia (1954) and Sharon and Katchalsky (1952) indicate that the equilibrium strongly favors the oxime. One of the ingredients of Plate Count Agar is glucose (0.1%), an aldehyde. Glucose can react with hydroxylamine to form an oxime (Jacobi, 1891). Thus one cannot exclude the possibility that the hydroxylamine liberated by the Arthrobacter sp. Q1 is combining with glucose (or a byproduct of algal metabolism) to form an oxime, and that this is the toxic principle in Chlorella inhibition. Indeed it can be observed in Figure 15 that acetaldoxime limited Chlorella growth, although not nearly as effectively as hydroxylamine. The equilibrium between an oxime and hydroxylamine, however, insures the presence of at least some of the latter, and the

toxicity observed on Chlorella plates containing the oxime may in fact have been due to this hydroxylamine. Amarger and Alexander (1968) concluded that their oxime apparently functioned as a constant source of hydroxylamine, which in turn was oxidized to nitrite. Jensen (1951), however, argued that oxime can be directly hydrolyzed to nitrite.

Evidence against oxime formation is, however, provided by comparing the hydroxylamine decay rate in BBM (Figure 23) with that in Plate Count Agar (Figure 18). They are similar. If oxime is indeed being generated, the oxime-hydroxylamine interaction in agar might be expected to result in a different decay rate than that found in BBM, which lacks ketones or aldehydes.

In our investigation, higher levels of glucose decreased the zone of inhibition, but did not eliminate it (see Table 4). This suggests that the glucose was stimulating bacterial growth which decreased the amount of nitrogen available for oxidation and liberation. Jensen (1951) found that glucose inhibited heterotrophic nitrification in liquid medium, and he also attributed this to stimulation of cell synthesis. A less likely alternative reason for the decreasing zone of inhibition with increasing glucose levels involves oxime formation. The greater the glucose concentration, the more oxime is formed. The consequent lower hydroxylamine level permits Chlorella growth closer to the Arthrobacter streak. The decrease in zone size is definitely

not due to a greater stimulation in Chlorella growth as a result of high glucose levels. Chlorella growth was actually markedly inhibited on agar plates at the 5% and 10% levels.

Besides hydroxylamine and oximes, other nitrogenous compounds cannot be excluded as the alga-inhibiting principle. The 1-nitropropane failed to inhibit Chlorella (Figure 15), but perhaps other nitro compounds could. Nitroso compounds were not tested for their ability to inhibit Chlorella. Certain N-nitroso substances are reported to be carcinogenic and mutagenic (Ayanaba and Alexander, 1974; Tate and Alexander, 1975), and indeed 20 µg/ml of a nitroso compound produced by Pseudomonas fragi inhibited Chlorella on agar plates (Tamura et al., 1967). According to Lund (1973) and Smith (1966), aliphatic primary and secondary nitroso compounds are unstable and tautomerize to oximes, often quite rapidly. It is noteworthy that Verstraete and Alexander (1973) discovered that 1-nitrosoethanol, a nitrification product from an Arthrobacter strain, was quite persistent in aqueous suspensions but not in soil. In addition, Murthy et al. (1966) found that a nitroso compound produced by Streptomyces alanosinicus was stable at a neutral pH.

Another type of compound which may arise from heterotrophic nitrification is a hydroxamic acid. Hydroxamic acids, which are very stable compounds (Smith, 1966), can be produced from the reaction of hydroxylamine with several other compounds (Siggia, 1963; Waelsch, 1952). Verstraete

and Alexander (1972a) detected this substance as a product of heterotrophic nitrification and found that the amount observed depended sharply on ferric iron levels in the medium. Approximately an order of magnitude difference in the yields of hydroxamic acid was noted between 0.0 and 0.1 µg/ml and between 0.1 and 1.0 µg/ml iron added. In our study, 2.0 µg/ml Fe^{+++} failed to decrease the zone of inhibition. Therefore, hydroxamic acid is evidently not involved in Chlorella inhibition.

Because of the low concentration of hydroxylamine found in lakes, the impact of bacteria which liberate this chemical may be sizable only when they are concentrated in regions rich in organic material. In such regions, these bacteria may affect the population dynamics of algal blooms.

One such region is sewage. Verstraete and Alexander (1973) found in large flasks containing raw sewage a pattern of nitrification resembling that of an Arthrobacter they had previously characterized. Hydroxylamine, which represented a major product in this pattern, was found in sewage at concentrations sufficient to inhibit Chlorella growth, as determined in our investigation. It is noteworthy that Chlorella is common in sewage and other highly polluted waters (Klotz et al., 1976; Eppley and MaciasR, 1963).

Our investigation has demonstrated that the population dynamics between an alga and a bacterium both common in lakewater depends upon the concentration of available organic

and inorganic nutrients. When nutrients are not plentiful, Chlorella inhibits Arthrobacter sp. Q1. When the nutrient supply is high, Arthrobacter inhibits Chlorella, at least when the latter is growing heterotrophically. This finding suggests that a bloom-forming alga may be controlled with the use of bacteria by the proper management of nutrients in the aquatic milieu.

RECOMMENDATIONS FOR FUTURE STUDY

1. The sensitivity of different algae, especially those which create noxious blooms, to low levels of hydroxylamine should be investigated. A related question of interest is the effect of hydroxylamine and its derivatives on the population dynamics of the planktonic ecosystem in general. Our study indicates the chemical may serve as a selective inhibitor against algae.

2. The manner in which the kinds and level of nutrient addition affects the relationship between Chlorella and the Arthrobacter (or a similar algal-bacterial nitrifier model) should be examined. We discovered that an increase in the concentration of organic material resulted in a reversal of the initial Chlorella inhibition of Arthrobacter. The type of organic nutrient also affects the relationship. Growth of Arthrobacter on Plate Count Agar yielded hydroxylamine, but growth on Nutrient Agar did not.

3. The fate of hydroxylamine in an ecosystem associated with algal blooms should be determined. Our study indicates that certain bacteria can degrade the substance, thereby rendering it ineffectual against algal cells.

4. The effect of high redox and high pH on the uptake kinetics of various freshwater bacteria and algae

should be ascertained. Our study suggests that competition for inorganic nutrients becomes more favorable for Chlorella relative to Arthrobacter as the pH rises.

5. The potential of algal cells in blooms to collectively liberate sufficient extracellular material to permit the production and liberation of detectable levels of hydroxylamine by heterotrophic nitrifiers should be clarified. We found evidence to the contrary in preliminary studies.

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A P P E N D I X A

Table 5. Specificity of Magee and Burris hydroxylamine test tested with various nitrogen compounds.

SAMPLE	SAMPLE-N TESTED (µg/ml)	MEDIA USED[1]		
		DISTILLED WATER	BROTH	PCA[2]
Potassium Nitrite	100	-	-	-
Hydroxylamine HCl	25	+	+	+
Acetaldoxime	400	+	+	+
Ammonium HPO_4	10,000	-	-	-
Sodium Nitrate	10,000	-	-	-
1-Nitropropane	400	-	-	-

[1]Tests in water and m-Plate Count Broth were conducted in tubes. Tests in Plate Count Agar were conducted in petri dishes.

[2]Sample-N tested was 0.1 that indicated.

Table 6. Specificity of Magee and Burris hydroxylamine test tested with various oximes.

SAMPLE	DETECTION EFFICIENCY (%)[1]		
	DISTILLED WATER	BROTH	PCA
Acetone Oxime	35	0.6	3
Acetaldoxime	7	0.3	1
2-Butanone Oxime	20	1.9	4
Hydroxylamine HCl	110	40.0	37

[1]$100 \times \frac{\text{[Sample-N] Detected}}{\text{[Sample-N] Added}}$.

A P P E N D I X B

Table 7. Chemical inhibitors of autotrophic nitrification and their effect on growth of Arthrobacter sp. Q1.

CHEMICAL	CONCENTRATION	Q1 GROWTH	PRODUCTION OF NH_2OH
Thiosemicarbazide	10^{-4}M	-	+
	5×10^{-5}M	+	+
$KClO_3$	8×10^{-2}M	+	+
1-Allyl-2-Thiourea	10^{-2}M	+	+
Ethylxanthic Acid (K salt)	10^{-3}M	-	
Hydrazine Hydrate	2×10^{-3}M	-	
Phenol	10^{-3}M	+	+
KCN	10^{-4}M	-	
	10^{-5}M	+	+
NaN_3	10^{-3}M	-	
	10^{-4}M	+	+

APPENDIX C

BOLD'S BASAL MEDIUM[1]

The following stock solutions are prepared and diluted as follows.

Macronutrients	Stock (g/l)	Vol. Used/l BBM
$NaNO_3$	25	10 ml
$CaCl_2 \cdot 2H_2O$	2.5	10 ml
$MgSO_4 \cdot 7H_2O$	7.5	10 ml
K_2HPO_4	7.5	10 ml
KH_2PO_4	17.5	10 ml
NaCl	2.5	10 ml
EDTA		1 ml
EDTA	50	
KOH	31	
Iron		1 ml
$FeSO_4 \cdot 7H_2O$	4.98	
H_2SO_4	1.0 ml	
Boron		1 ml
H_3BO_3	11.42	
Micronutrients		1 ml
$ZnSO_4 \cdot 7H_2O$	8.82	
$MnCl_2 \cdot 4H_2O$	1.44	
MoO_3	0.71	
$CuSO_4 \cdot 5H_2O$	1.57	
$Co(NO_3)_2 \cdot 6H_2O$	0.49	

Adjust to pH 6.6.

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[1]From Nichols and Bold (1965).

AMMONIUM-ACETATE MEDIUM[1]

Nutrient	g/l
KH_2PO_4	8.2
NaOH	1.6
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$(NH_4)_2SO_4$	4.7
Na acetate$\cdot 3H_2O$	16.9
$CaSO_4 \cdot 2H_2O$	0.0005
$CuSO_4 \cdot 5H_2O$	0.0005
$ZnSO_4 \cdot H_2O$	0.0005
$FeCl_3 \cdot 6H_2O$	0.0005
$NaHCO_3$	0.84

Adjust to pH 7.2.

[1]Revised from Verstraete and Alexander (1972a).

PLATE COUNT AGAR (DIFCO)

Nutrient	g/l
Bacto-Tryptone, Pancreatic Digest of Casein USP	5
Bacto-Yeast Extract	2.5
Bacto-Dextrose, Glucose	1
Bacto-Agar	15

pH 7.0 at 25°C.

A P P E N D I X D

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF SELECTED BACTERIA

The identification of Arthrobacter sp. Q1 (white organism), Flavobacterium sp. (yellow organism), and Nocardia sp. (red organism) was based upon the following criteria.

General Morphology

Arthrobacter sp. Q1: Small gram-negative rods changing to coccoid forms after several days. Motile with peritrichous flagella. Colonies were circular, smooth, moderately large, and cream-white.

Flavobacterium sp.: Gram-negative rods. Motile. Colonies were circular, glistening, raised, hard, large, and bright yellow.

Nocardia sp.: Small gram-negative rods; development of small filaments with some branching, but no aerial hyphae. Nonmotile. Nonacid-fast. Colonies were circular, smooth, small, and bright red.

Biochemical Tests	White	Yellow	Red
Acid and/or gas in Purple Broth Base	-	-	-
-sucrose	-	-	-
-maltose	-	-	-
-lactose	-	-	-
-glucose	-	-	-
-galactose	-	-	-
-fructose	-	-	-
-mannose	-	-	-
Acid in CTA			
-sorbitol	-	-	-
-dulcitol	-	-	-
-mannitol	-	-	-

Biochemical Tests	White	Yellow	Red
Growth in Simmons[1]			
citrate	-	-	-
succinate	+	+	-
pyruvate	+	-	-
acetate	+	+	-
Chitin hydrolysis	-	-	-
Starch hydrolysis	+	-	-
Liquification of gelatin	-	+	-
Catalase	+	+	+
Oxidase	+	-	-
Urease	+	-	-
Nitrate reduction	-	-	-
Indole	-	-	-
OF Test	st. aer.	st. aer.	-
Litmus Milk	sl. alk.	sl. P,R	-

[1]"Simmons" pyruvate, succinate, and acetate were prepared by combining the basic ingredients of Simmons Citrate Agar, except that salts of these organic acids were respectively substituted for the citrate.

KEY:
R = reduction of litmus milk
P = proteolysis of litmus milk
alk. = alkalinity in litmus milk
st. aer. = strict aerobe
CTA = Cystine Trypticase Agar

General Remarks

Nocardia failed to grow on most of the media used. It did grow in Purple Broth Base plus fructose, and on CTA. The *Flavobacterium* and *Arthrobacter* grew on most of the media, but failed to produce detectable amounts of acid or gas.

Figure 25a. An electron micrograph of a 24-hour culture of Arthrobacter sp. Q1 showing peritrichous flagella (1% ammonium molybdate; x 28,500).

Figure 25b. An electron micrograph of a 24-hour culture of Arthrobacter sp. Q1. Note the apparent side attachments of the majority of flagella (1% ammonium molybdate; x 84,000).


a

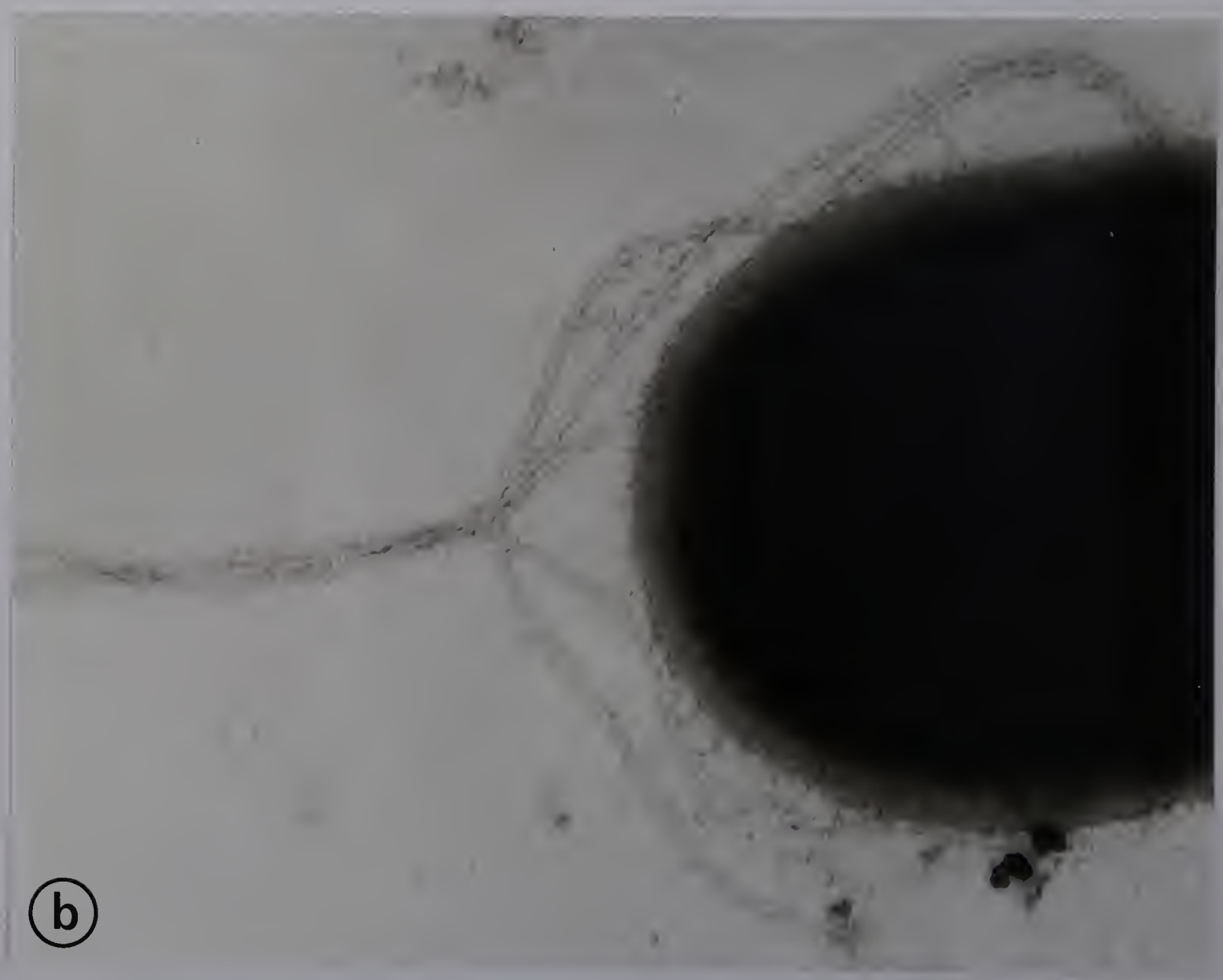
b

Figure 25c. An electron micrograph of a 48-hour culture of Arthrobacter sp. Q1 showing peritrichous flagella in both rod-shaped and coccoid forms (1% ammonium molybdate; x 14,400).

Figure 25d. An electron micrograph of a 48-hour culture of Arthrobacter sp. Q1 showing a flagellated coccoid form (1% ammonium molybdate; x 28,500).


c


d